University of London

Tania Rafid Adib

Gene expression ~~signatures in~~ MICROARRAYS OF ADENOCARCINOMA *serous ~~epithelial~~ ovarian ~~cancer~~*

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University College London

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Supervisors:

Professor Chris Boshoff

Dr Jonathan Ledermann

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

ABSTRACT

Ovarian cancer has the highest mortality rate of the gynaecological cancers. This is partly due to the lack of effective screening markers. In this study, oligonucleotide microarrays complementary to ~12,000 genes were used to establish a gene expression microarray (GEM) profile for normal ovarian tissue, as compared to stage III ovarian serous adenocarcinoma and omental metastases from the same individuals. The GEM profiles of the primary and secondary tumours from the same individuals were essentially alike, reflecting the fact that these tumours had already metastasised and acquired the metastatic phenotype. A novel biomarker, mammaglobin-2 (MGB2), was identified which is highly expressed specific to ovarian cancer. MGB2, in combination with other putative markers identified here, could have potential for screening.

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ABBREVIATIONS

AdC	adenocarcinoma
ADN	adipsin
AGRN	agrin
AKT	V-AKT murine thymoma viral oncogene homologue
ALL	acute lymphoid leukaemia
AML	acute myeloid leukaemia
APC	adenomatous polyposis of the colon
ARH1	ras homologue gene family, member 1
ATP	adenosine triphosphates
BAX	bcl2-associated X protein
BCL-2	b-cell CLL/lymphoma 2
bFGF	basic fibroblast growth factor
BLAST	basic local alignment search tool
bp	base-pair
BRCA1	breast cancer 1
BRCA2	breast cancer 2
BSA	bovine serum albumin
c-erbB-2	erb-b2 avian erythroblastic leukemia viral oncogene homologue 2
C-myc	myelomatosis viral oncogene homologue
CA	California
CA125	ovarian carcinoma antigen CA125
CCR	chemokine, cc motif, receptor
CD24	CD24 antigen
CD9	CD9 antigen
Cdc	cell division cycle
CDH	cadherin
CDK	cyclin-dependent kinase
CDKN2A	cyclin-dependent kinase inhibitor 2a
CDH1	E-cadherin
cDNA	complementary deoxyribonucleic acid
CGH	comparative genomic hybridisation
CLDN	claudin
COL3A1	collagen, type III, alpha-1
CP	ceruloplasmin
CXCR	chemokine, cxc motif, receptor
DAB	diaminobenzidine
dATP	2'-deoxyadenosine 5'-triphosphate
DCC	deleted in colorectal carcinoma
dCTP	2´-deoxycytidine 5´-triphosphate
DEPC	diethylene pyrocarbonate
dGTP	2´-deoxyguanosine 5´-triphosphate
DHEA	dehydroepiandrostenedione
DLBCL	diffuse large B-cell lymphoma
Doc 2	differentially expressed in ovarian cancer 2
DNA	deoxyribonucleic acid
dNTP	nucleotide

ds	double stranded
DTT	dithiothreitol
dTTP	2'-deoxythymidine 5'-triphosphate
E-cad	E-cadherin
E2F	E2F transcription factor
ECM	extracellular matrix
EDTA	ethylenediamineteraacetic acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EOC	epithelial ovarian cancer
EST	expressed tag sequences
EZH2	enhancer of zeste, drosophila, homolog 2
FDR	false discovery rate
FGF	fibroblast growth factor
FHL2	four-and-a-half lim domains 2
FIGO	Federation of Gynaecology and Obstetrics
FISH	fluorescence *in-situ* hybridisation
G0	Resting phase of the cell cycle
G1	GI gap of the cell cycle
G2	G2 gap of the cell cycle
GA733-1	tumour-associated calcium signal transducer 2; TACSTD2
GA773-2	tumour-associated calcium signal transducer 1; TACSTD1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GEM	gene expression microarray
GPCR	G-protein-coupled receptor family
H&E	haematoxylin and eosin
H_2O	water
HER	V-ERB avian erythroblastic leukemia viral oncogene homologue
Her-2/neu	ERB-B2
HNPCC	hereditary non-polyposis colorectal cancer
HPN	hepsin
HRT	hormone replacement therapy
HRAS	V-HA-RAS harvey rat sarcoma viral oncogene homologue
HS	heparan sulphate
Hyb	hybridisation
IFI-15K	interferon-induced protein 15
IGF	insulin-like growth factor
IgG	immunoglobulin
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IGL	immunoglobulin lambda locus
IHC	immunohistochemistry
IVT	*in vitro* transcription
K-ras	V-KI-RAS kirsten rat sarcoma viral oncogene homologue
KLK	kallikrein
KRT	keratin
LMNB1	lamin B1
LMP	low malignant potential
LOH	loss of heterozygosity
LPA	lysophosphatidic acid

LPL	lipoprotein lipase
LU	Lutheran group
MAPK	mitogen activated protein kinase
MAX	myc-associated factor X
MGB	mammaglobin
MGB2	mammaglobin B2
MLH1	colon cancer, familial nonpolyposis, type 2
MM	mismatch
MMAC1	mutated in multiple advanced cancers
MMP	matrix metalloproteinase
MLH1	colorectal cancer, hereditary nonpolyposis, type 2; hnpcc2
mRNA	messenger RNA
MSH2	mutS homologue 2
MUC-1	mucin-1
myc	v-myc avian myelocytomatosis viral oncogene homologue
NCBI	National Centre for Biotechnology Information
NME1	nonmetastatic cells 1 (nm23-H1)
NME2	nonmetastatic cells 2 (nm23-H2)
NTC	non template control
OCP	oral contraceptive pill
OPCML	opioid-binding cell adhesion molecule
OSE	ovarian surface epithelium
OSF2	runt-related transcription factor 2
PAI	plasminogen activator-inhibitor
PCNA	proliferative cell nuclear antigen
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PDGFRα	platelet-derived growth factor receptor α
PEG-3	paternally expressed gene-3
PI3-kinase	phosphatidylinositol 3-kinase
PIK3CA	phosphatidylinositol 3-kinase, catalytic, alpha
PLIN	perilipin
PM	perfect match
PMS1	Postmeiotic segregation increased 1, yeast homologue
PMS2	Postmeiotic segregation increased 2, yeast homologue
PRAME	preferentially expressed antigen in melanoma
pRB1	retinoblastoma protein
PRSS8	prostasin
PTEN	phosphatase and tensin homolog deleted on chromosome ten
PTTG1	pituitary tumour-transforming 1 interacting protein
PUMP1	matrix metalloproteinase-7 (MMP-7)
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
RhoC	ras homolog gene family, member C
RT	reverse transcription
RT-PCR	reverse transcriptase polymerase chain reaction
RNA	ribonucleic acid
rRNA	ribosomal RNA
runx2	runt-related transcription factor 2 (OSF2)
S phase	DNA synthesis phase of the cell cycle
SAA1	serum amyloid 1

SAGE	serial analysis of gene expression
SAPE	streptavidin-phycoerythrin
siRNA	small interfering RNA
SLPI	secretory leukocyte protease inhibitor
SOM	self-organising map
ss	single stranded
SSIIRT	SuperScript II reverse transcriptase
SPARC	secreted protein acidic and rich in cysteine
SPP1	secreted phosphoprotein 1
STK15	serine/threonine protein kinase 15
SVM	support vector machine
TACSTD1	tumour-associated calcium signal transducer 1 (GA773-2)
TAE	Tris-acetate-EDTA
TEPI	TGFβ-regulated and epithelial cell-enriched phosphatase
TGF	transforming growth factor
TGF-α	transforming growth factor-α
TGF-β	transforming growth factor-β
TGFR	transforming growth factor receptor
TJ	tight junction
TVS	transvaginal ultrasound
TIMP	tissue inhibitor of metalloproteinase
tRNA	transfer RNA
UK	United Kingdom of Great Britain and Northern Ireland
UKCTOCS	UK Collaborative trial of ovarian cancer screening
u-PA	urokinase-type plasminogen activator
uPAI1	urokinase-type plasminogen activator inhibitor
uPAR	urokinase-type plasminogen activator receptor
UV	ultraviolet
VEGF	vascular endothelial growth factor
VEGF-C	vascular endothelial growth factor-C
VEGF-D	vascular endothelial growth factor-D
VEGFR-1	vascular endothelial growth factor receptor-1
VEGFR-2	vascular endothelial growth factor receptor-2
VEGFR-3	vascular endothelial growth factor receptor-3
WHI	Women's Health Initiative
WISP-2	wnt-inducible signalling protein-2

CHAPTER 1

INTRODUCTION

Ovarian cancer is the fourth most common cancer in women, after breast, colorectal and lung cancers. The UK has one of the highest incidences of ovarian cancer in Europe, with around 6,800 new cases being diagnosed each year, and 4,000 deaths [Cancer Research UK, 2002]. In America, ovarian cancer accounts for 4 percent of all cancers among women and ranks fifth as a cause of their deaths from cancer. The American Cancer Society statistics for ovarian cancer predicted that there would be approximately 25,400 new cases and 14,300 deaths in 2003 [Jemal et al., 2003]. 70% of women present at an advanced FIGO (International Federation of Gynaecology and Obstetrics) stage, and overall 60% of patients with ovarian cancer will die from their disease. The high mortality of ovarian cancer is due firstly to the fact that the disease is relatively asymptomatic in its early stages, and that the symptoms of late stage disease, such as abdominal discomfort, weight loss, diarrhoea or constipation, vaginal bleeding and shortness of breath, are non-specific complaints. Secondly, ovaries are inaccessible pelvic organs, and therefore effective screening methods for early stage disease have remained elusive. Finally, no pre-malignant phenotype has been identified which is known to proceed through a stepwise progression to cancer which is amenable for screening. Survival figures for epithelial ovarian cancer (EOC), when diagnosed in its earliest stages, give rates of greater than 90%. However, stage IV disease has a 5 year survival of around 15% with overall 5 year survival for all four stages of 25%. This has remained largely unchanged over the past 20 years, despite new chemotherapeutic agents.

The recent availability of gene expression microarrays has transformed the study of cancer biology and has enabled the simultaneous examination of thousands of genes in parallel. This promises to extend our knowledge of the molecular events in the evolution and progression of ovarian cancer, extend cancer classification and identify molecules for effective prevention, detection and treatment of this disease. This thesis concerns the use of gene expression microarrays to identify novel markers in ovarian cancer which may contribute to this knowledge.

1.1 Classification of Ovarian Tumours

The classification of all ovarian tumours is based on the tissue of origin. This is best understood in terms of the embryology of the ovary. The development can be divided into four main stages. First, the primordial germ cells become detached from the endoderm of the yolk sac wall and migrate to the genital ridges, which are bilateral thickenings of coelomic epithelium. Second, the coelomic epithelium undergoes proliferation, along with the underlying mesenchyme. During the third stage, the ovary divides into a central medulla and a peripheral cortex. The fourth stage comprises development of the cortex and involution of the medulla. The histogenetic classification categorises ovarian neoplasms according to whether they originate from the coelomic epithelium, germ cells or mesenchyme.

1.1.1 Histogenetic Classification of Ovarian Neoplasms

The histological classification of ovarian tumours by the World Health Organisation (WHO) is based on histogenetic principles, that is, ovarian tumours are characterised according to whether they are derived from coelomic surface epithelial cells, germ cells, or mesenchyme (the stroma and the sex cord). Epithelial ovarian tumours, which constitute 85-90% of malignant ovarian tumours, are further classified into histological types as follows: serous, mucinous, endometrioid, clear cell, transitional cell tumours (Brenner tumours), carcinosarcoma, mixed epithelial tumour, undifferentiated carcinoma, and others. Carcinosarcoma of the ovary, also known as malignant mixed mesodermal tumour (MMMT) behaves in a different way to other epithelial ovarian cancers in that it has a later age of onset, worse response to platinum-based chemotherapy and overall poor prognosis [Brown et al., 2004]. Clear cell and endometrioid carcinomas are highly associated with endometriosis. In stage distribution, serous carcinoma is found predominantly is stage III or IV. In contrast, clear cell and endometrioid carcinomas tend to remain confined to the ovary. Clear cell and endometrioid carcinomas may be unique histological types compared with serous carcinomas with respect to stage distribution and association with endometriosis.

Classification of Ovarian Tumours

Tumours Derived from Coelomic Epithelium

- Serous tumour
- Mucinous
- Endometrioid
- Clear cell
- Brenner
- Undifferentiated
- Carcinosarcoma & mixed mesodermal

Tumours Derived from Germ Cells

- Teratoma
- Dysgerminoma
- Embryonal carcinoma
- Endodermal sinus tumour
- Choriocarcinoma
- Gonadoblastoma

Tumours Derived from Gonadal Stroma

- Granulosa-theca cell tumours
- Sertoli-Leydig tumours

It is clear from the above list that the ovary is the origin of a great number of tumour types. By far the majority (85-90%) of all malignant ovarian cancers are epithelial in origin, and are therefore referred to as epithelial ovarian cancers. The most common (~60%) of the epithelial tumours are the serous cystadenocarcinomas.

Serous cystadenocarcinoma	60%
Endometrioid carcinoma	20%
Mucinous cystadenocarcinoma	10%
Undifferentiated carcinoma	8%
Clear Cell carcinoma	2%

Table 1.1. Percentage of epithelial ovarian cancer by subtypes

1.2 Epidemiology

Epithelial ovarian cancer (EOC) is a disease of perimenopausal and postmenopausal women. The mean age of diagnosis is between 50 and 70 years of age. 90% of all EOC's are thought to be sporadic, and 10% are familial.

1.2.1 Hereditary Epithelial Ovarian Cancer

A family history of ovarian cancer accounts for the greatest of all known risk factors, other than age, for the disease [Parazzini et al., 1991], with about 10% of all EOC's resulting from a hereditary predisposition [Claus et al., 1996]. This thesis is not concerned with hereditary ovarian cancer, so it is only briefly mentioned.

1.2.1.1 The Breast and Ovarian Cancer Syndrome

The breast and ovarian cancer syndrome is responsible for about 90% of all cases of hereditary ovarian cancer [Narod et al., 1995a]. Approximately 10% of these women are carriers of the breast/ovarian cancer susceptibility genes, BRCA1 and BRCA2 [Risch et al., 2001]. The lifetime risk of EOC in the general population is about 1.4%, while that for gene carriers, such as Ashkenazi Jews ranges between 16-30% [Struewing et al., 1997;Claus et al., 1996;Whittemore et al., 1997]. Families who have a total of five or more breast or ovarian cancers in first or second degree relatives are thought to qualify, as are families where at least three relatives have early-onset (less than 60 years) breast or ovarian cancer [Narod et al., 1995b]. Both BRCA1 and BRCA2 are tumour suppressor genes and are transmitted in an autosomal dominant fashion.

The BRCA1 gene, located on chromosome 17q, is associated with an increased risk of both ovarian and breast cancers, with a lifetime risk of ovarian cancer of 20-40% [Whittemore et al., 1997;Ford et al., 1994]. BRCA1 mutations are responsible for 5.7% of ovarian cancers in women under the age of 40 years in the general population, 4.6% between the ages of 40 and 50, and 1.1% above the age of 50 [Ford et al., 1995]. Features suggestive of a BRCA1 mutation include a family history

of: two or more cases of ovarian cancer, ovarian and breast cancer in the same woman, one or more cases of pre-menopausal breast cancer with a case of ovarian cancer diagnosed at any age, two or more cases of postmenopausal breast cancer and one or more cases of ovarian cancer diagnosed at any age or male breast cancer.

The BRCA2 gene on chromosome 13q is also associated with high rates of ovarian and breast cancer. The lifetime risk of breast cancer has been reported to be similar to that of BRCA1 (55-85%), while the lifetime risk of ovarian cancer is estimated to be 10-20% [Ford et al., 1998]. BRCA2 is also associated with up to 40% risk of male breast cancer [Couch et al., 1996], as well as an increased risk of pancreatic cancer [Hahn et al., 2003]. BRCA2 features are similar to those outlined for BRCA1, but also include a family history of pancreas cancer in addition to breast and/or ovarian cancer. The role of the BRCA1 and BRCA2 genes in sporadic ovarian cancer remains unclear, since somatic mutations in either gene are uncommon. However, recently mutations in EMSY were identified in 17% of high-grade ovarian cancers [Hughes-Davies et al., 2003]. EMSY maps to chromosome 11q13.5, a region known to be involved in breast and ovarian cancers. EMSY encodes a protein, EMSY, which binds to, and silences the function of BRCA2. This implicates the BRCA2 pathway of tumour suppression in sporadic ovarian cancer.

1.2.1.2 Hereditary Nonpolyposis Colorectal Cancer Syndrome

Hereditary Non-Polyposis Colorectal Cancer (HNPCC), also known as Lynch II syndrome is a hereditary syndrome most commonly characterized by an increased risk of colorectal, endometrial and ovarian cancers. It is closely linked with mutations in the DNA mismatch repair genes MSH2, MLH1, PMS1, and PMS2 [Lynch and Smyrk, 1996]. The lifetime risk for colorectal cancer is 80%, for endometrial cancer approximately 40%, and for ovarian cancer is 10% [Aarnio *et al.*, 1995]. Other associated cancers include stomach, small bowel, urinary tract, and biliary tract.

1.2.1.3 Site-Specific Ovarian Cancer

It was previously thought that families with an excess of ovarian cancer but no incidences of breast cancer form a third distinct group. However, genetic linkage analyses have only demonstrated linkage to BRCA1 [Steichen-Gersdorf *et al.*, 1994], suggesting that these families are a variant of the breast and ovarian cancer syndrome where early-onset breast cancer is rare or has not yet appeared. There is a fourth group, comprising very young (less than 30 years old) women who have invasive ovarian cancer, but one recent study demonstrated that this is unlikely to be due to genetic predisposition [Stratton *et al.*, 1999].

1.2.2 Sporadic Ovarian Cancer

90% of EOC is sporadic. The precise aetiology is not known, but several environmental, dietary and hormonal causes are suggested. These are detailed here.

1.2.2.1 Country of Origin, Race and Age

Epithelial ovarian cancer is predominantly a cancer of the developed world. Approximately 190,000 new cases and 114,000 deaths occur worldwide annually.

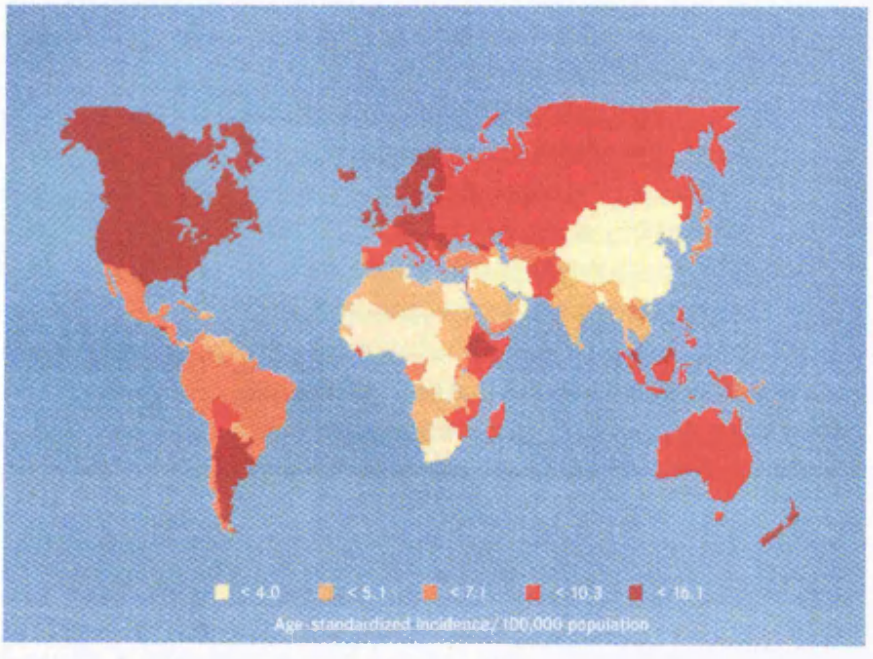


Figure 1.1. Incidence of ovarian cancer worldwide. Figure taken from WHO, World Health Report.

The highest rates are found in the countries of Northern and Western Europe, USA and Canada. The lowest rates are found in Africa and Asia (see Figures 1.1 and 1.2).

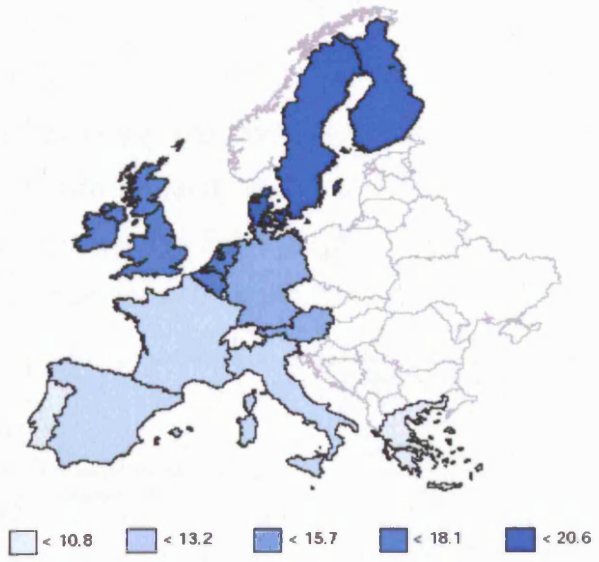


Figure 1.2. Age-standardised incidence rates for ovarian cancer (per 100,000 females per year) in the European Union, 1995.
Figure taken from Ferlay *et al*, 1999. Cancer incidence, mortality and prevalence in the European Union.

Survival differs significantly throughout the world. (Figure 1.3). It is unclear why this is the case.

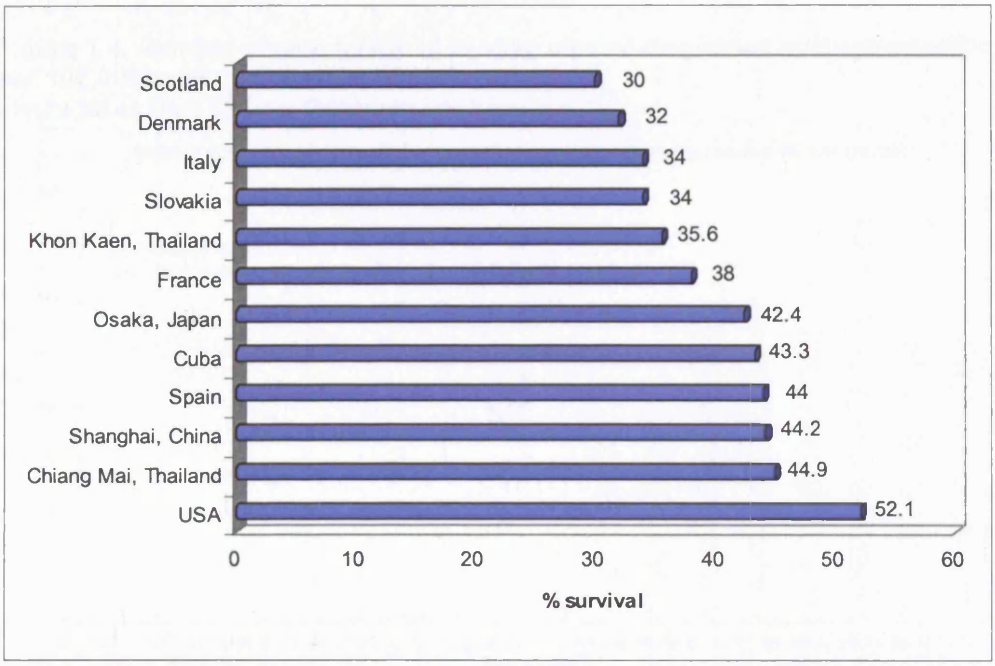


Figure 1.3. Five-year relative survival rates after diagnosis of ovarian cancer.
Data adapted from World Health Organisation, World Health Report.

Ovarian cancer is a disease of increasing age. This is demonstrated by incidence and mortality rates for the UK (Figures 1.4 and 1.5). The incidence remains low until the age of 40 years and rises dramatically thereafter, with a peak in the seventh decade.

Women who migrate from countries with a low risk for ovarian cancer such as Asia to high-risk areas such as North America, there is a gradual increased incidence of ovarian cancer in these women compared to the expected rates for native born women [Kliewer and Smith, 1995].

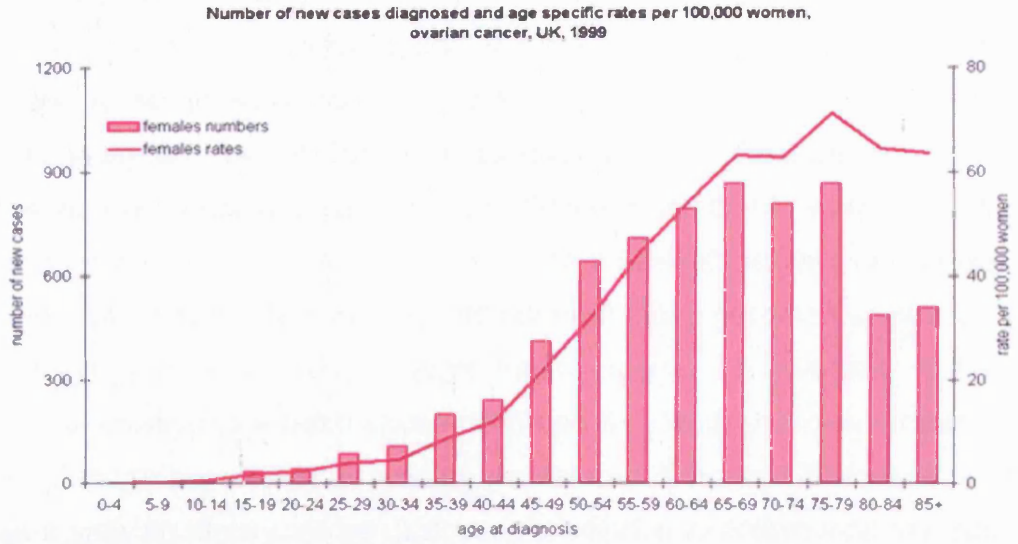


Figure 1.4. Number of new cases of ovarian cancer diagnosed and age-specific rates per 100,000 women in the UK, 1999.
Figure taken from Cancer Research UK Statistics

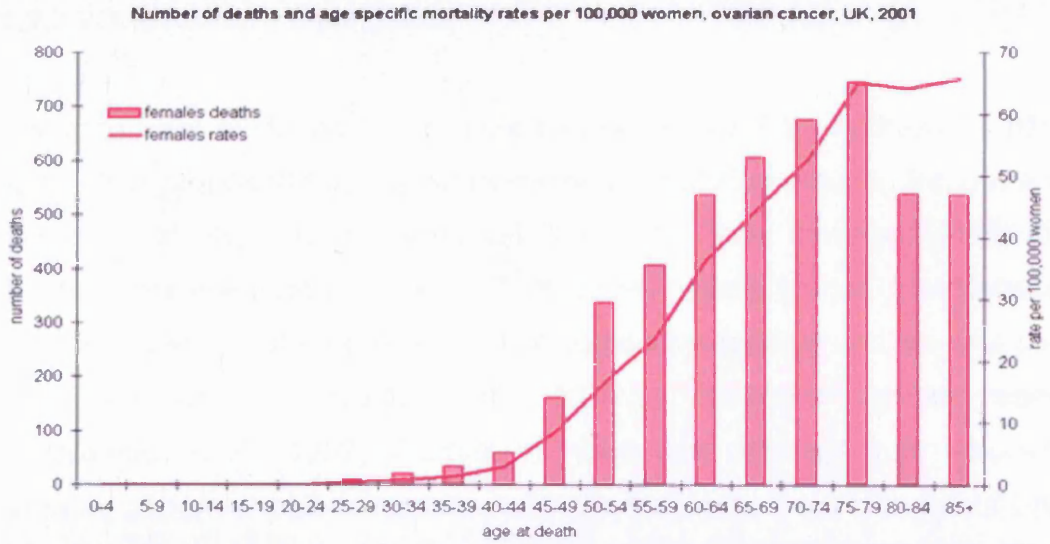


Figure 1.5. Number of deaths from ovarian cancer and age-specific mortality rates per 100,000 women in the UK, 1999. Figure taken from Cancer Research UK Statistics

1.2.2.2 Aetiology of Sporadic Epithelial Ovarian Cancer

Epidemiological studies have consistently shown that the risk of ovarian cancer is reduced by factors which inhibit ovulation, such as pregnancy, breast feeding and the combined oral contraceptive pill. Two major theories emerged; that of incessant ovulation and gonadotrophin stimulation. The incessant ovulation hypothesis is based on repeated damage and repair of the ovarian surface epithelium leading to increased epithelial cell proliferation and an accumulation of genetic mutations, and ultimately to tumour formation. The gonadotrophin hypothesis postulates that the pituitary FSH/LH surges cause elevations in oestrogen and these high levels cause stimulation of the ovarian epithelium leading to neoplastic transformation. Although there is some evidence in favour of these hypotheses, there are major flaws principally with relation to the magnitude of their effect being disproportionate with the increased risk of ovarian cancer. One unifying theory which has been proposed is inflammation. Inflammatory agents such as asbestos, talc and endometriosis have been linked to ovarian cancer, and although have not been shown to be causal, may indirectly have a role through inflammatory mechanisms. Tubal ligation and hysterectomy have been shown to be protective for ovarian cancer, and again may act through preventing ascending genital tract infection. The process of inflammation as a unifying theory will be discussed in relation to aetiological risk factors for ovarian cancer.

1.2.2.2.1 Incessant Ovulation

The incessant ovulation theory was first put forward by Fathalla in 1971[Fathalla, 1971]. It is proposed that repeated cycles of ovulation-induced trauma and repair of the OSE at the site of ovulation leads to DNA damage which DNA repair mechanisms are unable to repair. This theory initially gained much support through studies showing that pregnancy, prolonged breast feeding and the oral contraceptive pill which suppress ovulation are strikingly protective against ovarian cancer [Whittemore et al., 1992]. Further evidence was obtained from laboratory studies showing abnormal p53 expression; p53 plays a central role in repair of DNA-damage apoptosis [Murdoch et al., 2001;Verschraegen et al., 2003]. However, the protection

is disproportionate as 5 year oral contraceptive usage gives a 50% risk reduction whilst only reducing the lifetime number of ovulations by 10-20% [Vessey and Painter, 1995]. In addition, there is evidence that the suppression of ovulation by pregnancy, breast feeding or the OCP has a greater effect in terms of risk reduction compared to the same length of suppression due to a late menarche or early menopause [Whittemore et al., 1992]. Although it is true that the extremes of menstrual life may be anovulatory, this further gives doubt as to whether it is purely the prevention of ovulation which is at play, or whether there is another process to account for this effect.

1.2.2.2.2 Gonadotrophins

The gonadotrophin theory proposes stimulation of the ovarian epithelium within inclusion cysts by high levels of oestrogen caused by high FSH and LH levels leads to ovarian cancer [Cramer and Welch, 1983]. As pregnancy provides the greatest overall risk reduction [Tung et al., 2005] and is associated with low basal levels of gonadotrophins, it was suggested that this was the mechanism by which the protective effect was conferred. However, pregnancy is also associated with very high levels of oestrogen, so this theory cannot hold. Other evidence that refutes this theory is the lack of correlation of serum levels of gonadotrophins with risk of developing ovarian cancer [Helzlsouer et al., 1995].

1.2.2.2.3 Infertility

It would follow from the incessant ovulation theory that anovulatory infertility is protective against ovarian cancer due to a lack of ovulation. There has been an ongoing debate regarding the link between ovarian cancer and infertility since it was first suggested in 1974 [Joly et al., 1974]. One study compared anovulatory infertility with other causes of infertility and found an relative risk of 1.8 [Rossing et al., 1994]. More recent studies have shown that it is not the drugs used per se, rather that nulliparous women with unexplained infertility in whom assisted conception was unsuccessful, had an inherently higher risk [Venn et al., 1999;Doyle et al., 2002].

1.2.2.2.4 Hormone Replacement Therapy

The use of hormone replacement therapy (HRT) has increased dramatically over the past 10 years. Several studies have investigated the possible links of HRT with cancer. Three studies [Lacey, Jr. et al., 2002;Riman et al., 2002;Purdie et al., 1999] suggest the risk of EOC with HRT use only applies to the use of unopposed oestrogens, with the risk increasing with the duration of use: odds ratio 1.8 (95% CI 1.1-3.0) between 10 and 19 years of use, increasing to an odds ratio of 3.2 (95% CI 1.7-5.7) after 20 years [Lacey, Jr. et al., 2002]. More recently, the women's health initiative (WHI) trial [Anderson et al., 2003] reported on 16,600 postmenopausal women with an intact uterus taking either placebo or combined oestrogen and progesterone HRT. Women without a uterus were also recruited, and were given either placebo or unopposed oestrogen. The first trial was stopped early in 2002 because an increased rate of breast cancers was found in HRT users, which outweighed the benefits of therapy. An increased rate of 58% for ovarian cancer was found, which was not significant. 27 ovarian cancers per 100,000 women were diagnosed in the placebo group compared with 42 per 100,000 in the treatment arm. There was no difference in tumour anatomy, stage or grade of disease between the two groups. The second trial is still ongoing and is expected to complete in 2005, and should provide information on the effect of unopposed oestrogen use on ovarian cancer.

Overall, HRT has not been found to increase the risk of ovarian cancer. However, one study has demonstrated an increased risk of endometrioid and clear cell epithelial ovarian cancers with the use of unopposed oestrogen [Garg et al., 1998].Postmenopausal HRT use causes increased levels of oestrogen with concomitant decreased levels of gonadotrophins, so would further argue against the gonadotrophin hypothesis.

1.2.2.2.5 Talc and Asbestos

In the 1960's it was noted that occupational exposure to asbestos was associated with the development of abdominal mesotheliomas, histologically similar to invasive

epithelial ovarian cancer [KEAL, 1960;Newhouse et al., 1977]. Talc (hydrated magnesium trisilicate) is a compound chemically related to asbestos, so a link between talc and EOC was suggested. Scientific evidence exists to demonstrate that asbestos and talc particles can travel to the upper genital tract in the presence of patent fallopian tubes [Heller et al., 1996a;Heller et al., 1996b;Venter, 1981]. Women using talc for perineal dusting and on sanitary napkins have a relative risk of invasive epithelial ovarian cancer of up to 3.28 ($p<0.001$) compared to women not performing either practices [Cramer et al., 1982]. The role of talc as a causative agent, however, is not universally accepted, as evidence to the contrary has been given [Wehner, 2002;Wong et al., 1999;Shen et al., 1998].

1.2.2.2.6 Tubal Ligation and Hysterectomy

Tubal ligation has been associated with a reduction in the risk of ovarian cancer with odds ratios of 0.2 to 0.9 [Green et al., 1997;Rosenblatt and Thomas, 1996]. The protective effect of hysterectomy without oophorectomy has shown similar risk reduction with odds ratios ranging between 0.03 and 0.8 [Loft et al., 1997]. This could be due to ovaries being inspected at the time of surgery and removed if they look abnormal. However, some studies have shown that the protective effect is sustained over 20-25 years [Cramer and Xu, 1995]. It has been suggested that this may be due to the prevention of inflammatory and potentially carcinogenic agents, such as talc, ascending the genital tract. Interestingly, one study showed a 50% decreased risk of ovarian cancer in women using talc but having had a sterilisation compared with a 30% increased risk in those using talc but not having been sterilised [Whittemore et al., 1989]. The same study found no protective effect of hysterectomy in women with a previous tubal ligation. The magnitude of the protection form the effects of talc are greater than expected, which suggests that other agents may also ascend the genital tract and cause inflammation, such as sexually transmitted infections.

1.2.2.2.7 Pelvic Inflammatory Disease

Pelvic inflammatory disease (PID) is an inflammation of the ovaries, fallopian tubes and endometrium due to sexually transmitted infections which ascend the genital

tract. An association between PID and ovarian cancer has been found, with the risk increasing proportionately with the number of infective episodes [Shu et al., 1989;Risch and Howe, 1995]. These women were more likely to have had PID at an early age, be nulliparous and infertile. PID causes inflammation and damage to the fallopian tube epithelium, and thereby tubal infertility; in fact women with tubal infertility have been reported to have a 3 fold risk of ovarian cancer [Rossing et al., 1994]. Therefore the association between PID and ovarian cancer lends further support for an inflammatory role in the causation of this disease.

1.2.2.2.8 Endometriosis

Endometriosis has been suggested to be a possible premalignant lesion of ovarian cancer, as a high proportion of endometrioid and clear cell cancers have associated endometriosis adjacent to malignant cells [Heaps et al., 1990]. In addition, endometriosis is associated with decreased fertility and general endocrine dysfunction, both of which could be important in ovarian carcinogenesis. Endometriosis is characterised by the presence of endometrium in places other than the lining of the uterus, most often the ovaries. This causes a local inflammatory reaction, with the presence of macrophages and inflammatory cytokines. This again suggests that inflammation may have a role in ovarian cancer.

1.2.2.2.9 The Unifying Role of Inflammation in Ovarian Cancer

The many risk factors detailed above have led to a number of different theories on the overall mechanism causing ovarian cancer. These include incessant ovulation, high gonadotrophin levels and direct hormonal effects. It has been proposed that inflammation may be the key mechanism which underlies the effects of the different exposures, and is discussed below.

The role of inflammation may be mediated by the production of toxic oxidants by the process of inflammation causing direct DNA damage. This damage may then lead to cancer. DNA mutations occur more frequently in rapidly dividing cells where errors in replication and repair are not corrected; chronic inflammation is associated with a

high cell turnover, so DNA integrity may be affected.

There is evidence that inflammatory molecules are increased in ovarian cancer. Chemokines, cytokines, adhesion molecules and other components of the extracellular matrix may contribute to a tissue environment that supports tumour proliferation and invasion. Chemokines have been found in ovarian cancers and ascites [Negus et al., 1995], and are known to facilitate the migration of immune cells into the tumour environment. Prostaglandins which are also mediators of inflammation are found in elevated amounts in ovarian cancers compared to normal ovaries [Gubbay et al., 2005]. Non-steroidal anti-inflammatory medications which reduce prostaglandin levels and have been found to be associated with a reduced risk of epithelial cancers including ovarian, breast and colon cancers [Harris et al., 2005]. This lends weight to the inflammation theory.

Ovulation itself may predispose to carcinogenesis. The process of follicle rupture and subsequent invagination of epithelial cells around the edge of the wound can lead to entrapment of inclusion cysts. These cysts have been shown to be present in high frequency in women with ovarian cancer in the contralateral unaffected ovary. They are also more common in the ovaries of women who have a high genetic predisposition to ovarian cancer. These women may have ovarian surface epithelia which are already predisposed to ovarian cancer and one feature of this predisposition is the presence of inclusion cysts. P53 mutations are a frequent finding in ovarian cancer and are thought to be due to spontaneous errors in DNA synthesis [Jones et al., 1991]. One study has shown that ovarian cancers which have a p53 mutation are associated with an increase in the number of lifetime ovulations [Schildkraut et al., 1997]. However, this has not been replicated by others [Webb et al., 1998]. The carcinogenic effect of ovulation may be mediated by inflammation. There is a significant elevation of inflammatory markers around the ovulatory follicles [Espey, 1994] which affects the surrounding epithelium causing cellular oxidative damage and thereby leading to mutagenesis.

In summary, ovulation, endometriosis, PID and talc can induce inflammation and increase the risk of ovarian cancer. Conversely, sterilisation and hysterectomy

protect the ovaries from the effect of substances which cause inflammation and thereby reduce the risk. The process of inflammation causes DNA damage and repair, oxidative stress, an increase in inflammatory molecules, all of which may be carcinogenic. Inflammation may be a unifying theory to explain the epidemiological risk factors for ovarian cancer although more laboratory studies are required for confirmation.

1.3 Pathology of Serous Cystadenocarcinomas

1.3.1 Histopathology

Each epithelial ovarian cancer subtype resembles its tissue of origin. Serous cystadenocarcinomas resemble cells of the fallopian tube, mucinous tumours resemble the cervical cells, endometrioid tumours the endometrium and clear cell tumours the transitional cell epithelium of the bladder.

Serous cystadenocarcinomas are the most commonly encountered histologic type, comprising just over 40% of all primary ovarian neoplasms. Approximately 30-50% are malignant and 35-50% of tumours are bilateral. They tend to be large tumours with multiple and cystic spaces containing friable papillary projections, and solid nodules of adenocarcinoma (Figure 1.6). The fluid within the cysts tends to be watery and thin or "serous" in nature. At a microscopic level, psammoma bodies are frequently seen; these are small, laminated calcospherites composed of microcrystals similar to calcium-phosphate apatite crystals of bone, and are produced intracellularly. They are believed to be a consequence of dystrophic calcification associated with cellular degeneration. Tumours containing psammoma bodies (Figure 1.7) have an above-average survival, possibly because of their diploid DNA content and low S-phase fraction [Kuhn *et al.*, 1989].

Serous adenocarcinomas can be classified according to their grade, with grade I (well-differentiated) tumours being composed almost entirely of glands and papillae, grade III (poorly-differentiated) almost entirely of sheets of malignant cells, with grade II containing a mixture of the two. Grading of tumours is very important as this confer varying prognoses. Median survival times for patients with grades I, II and III tumours are 4.6, 2.3 and 1.5 years, respectively [Demopoulos *et al.*, 1984].

For this thesis I only focused on serious carcinoma, because if I included all different histological subtypes, I would have had to collect enough samples from the each group and the overall project would have been too complicated to complete within 24 months. I used serous ovarian adenocarcinomas for my study, although these may

have in fact been fallopian tube cancers. This possible divergence stems from the fact that more than 95% of fallopian tube cancers are papillary serous adenocarcinomas and have the same microscopic appearance as the serous epithelial ovarian cancers. The diagnosis of a fallopian tube cancer requires that the major portion of the cancer is within the tube rather than on the ovary. This was the case with all cancers I used for my experiments.

Figure 1.6. Macroscopic appearance of serous ovarian adenocarcinoma.
Most often, at advanced stages, the entire ovary is taken over by tumour and no normal ovarian tissue is recognisable.

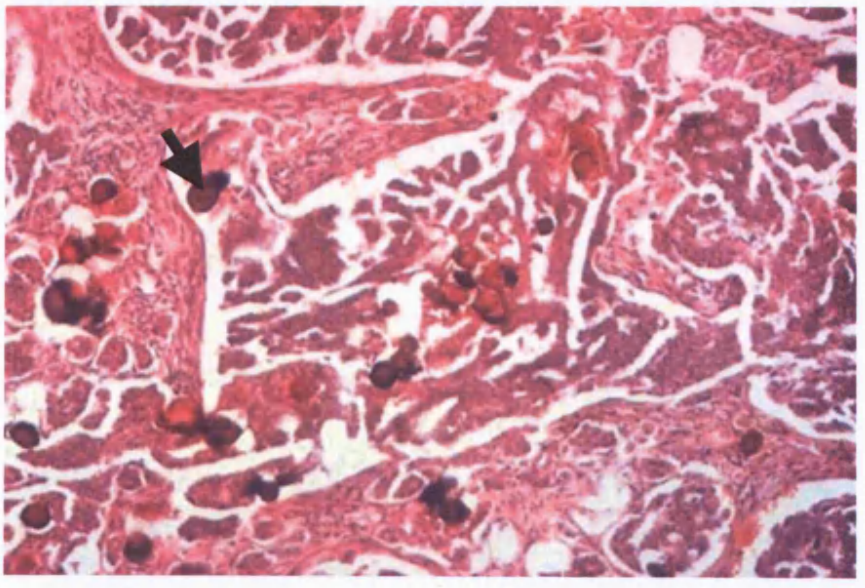


Figure 1.7. Microscopic appearance.
There are sheets of cancer cells with focal calcification (psammoma bodies, arrow).

1.3.2 Staging

Ovarian cancer stage is the most important prognostic factor and, in advanced stage patients, volume of residual disease. The International Federation of Gynecology and Obstetrics (FIGO) has standardised the staging of gynaecological cancers (see Figure 1.8). FIGO stage is such a powerful predictor of prognosis in ovarian cancer that most other putative prognostic factors are of little importance in comparison to stage. Staging takes both surgical and pathological findings into account, hence the term, "surgicopathologic stage".

FIGO stage I ovarian carcinomas have an excellent prognosis. These patients have a 5-year survival of over 90%, as do patients in stages IA and IB (Figure 1.8). Poor prognostic factors in stage I include grade 3 histology and IC substage, both of which are associated with poorer survival rates. Although it is possible that the IC substage based on malignant cells in ascites or peritoneal washings is the first evidence of true metastatic ability, these cells may merely be exfoliated.

Stage II ovarian cancer is a small and heterogeneous group, and makes up about 10% of ovarian cancers. It is defined as extension or metastasis to extraovarian pelvic organs, most commonly the fallopian tubes and pelvic peritoneum.

Stage III is most commonly encountered at presentation (at least 50% of cases). Tumour spreads along peritoneal surfaces, in the peritoneal fluid along the paracolic gutters and to the right subdiaphragmatic space. Metastases spread to the retroperitoneal lymph nodes and less commonly to the inguinal lymph nodes. It is still unclear whether omental and peritoneal spread is due to direct contiguous spread, or whether it is due to lymphatic or vascular spread.

Stage IV includes metastatic spread to the liver parenchyma and accounts for 13% of cases. The liver and lungs are the most common metastatic sites. Brain metastases are only present in 0.1% of patients at presentation.

Carcinoma of the ovary: FIGO nomenclature (Rio de Janeiro 1988)

Stage I Growth limited to the ovaries

- Ia Growth limited to one ovary; no ascites present containing malignant cells. No tumour on the external surface; capsule intact
- Ib Growth limited to both ovaries; no ascites present containing malignant cells No tumour on the external surfaces; capsules intact
- Ic * Tumour either Stage Ia or Ib, but with tumour on surface of one or both ovaries, or with capsule ruptured, or with ascites present containing malignant cells, or with positive peritoneal washings

Stage II Growth involving one or both ovaries with pelvic extension

- IIa Extension and/or metastases to the uterus and/or tubes
- IIb Extension to other pelvic tissues
- IIc * Tumour either Stage IIa or IIb, but with tumour on surface of one or both ovaries; or with capsule(s) ruptured; or with ascites present containing malignant cells or with positive peritoneal washings

Stage III Tumour involving one or both ovaries with histologically confirmed peritoneal implants outside the pelvis and/or positive retroperitoneal or inguinal nodes.
Superficial liver metastases equals Stage III. Tumour is limited to the true pelvis, but with histologically proven malignant extension to small bowel or omentum

- IIIa Tumour grossly limited to the true pelvis, with negative nodes, but with histologically confirmed microscopic seeding of abdominal peritoneal surfaces, or histologically proven extension to small bowel or mesentery
- IIIb Tumour of one or both ovaries with histologically confirmed implants, peritoneal metastasis of abdominal peritoneal surfaces, none exceeding 2 cm in diameter; nodes are negative
- IIIc Peritoneal metastasis beyond the pelvis > 2 cm in diameter and/or positive retroperitoneal or inguinal nodes

Stage IV Growth involving one or both ovaries with distant metastases. If pleural effusion is present, there must be positive cytology to allot a case to Stage IV.
Parenchymal liver metastasis equals Stage IV

* In order to evaluate the impact on prognosis of the different criteria for allotting cases to Stage Ic or IIc, it would be of value to know if rupture of the capsule was spontaneous, or caused by the surgeon; and if the source of malignant cells detected was peritoneal washings, or ascites.

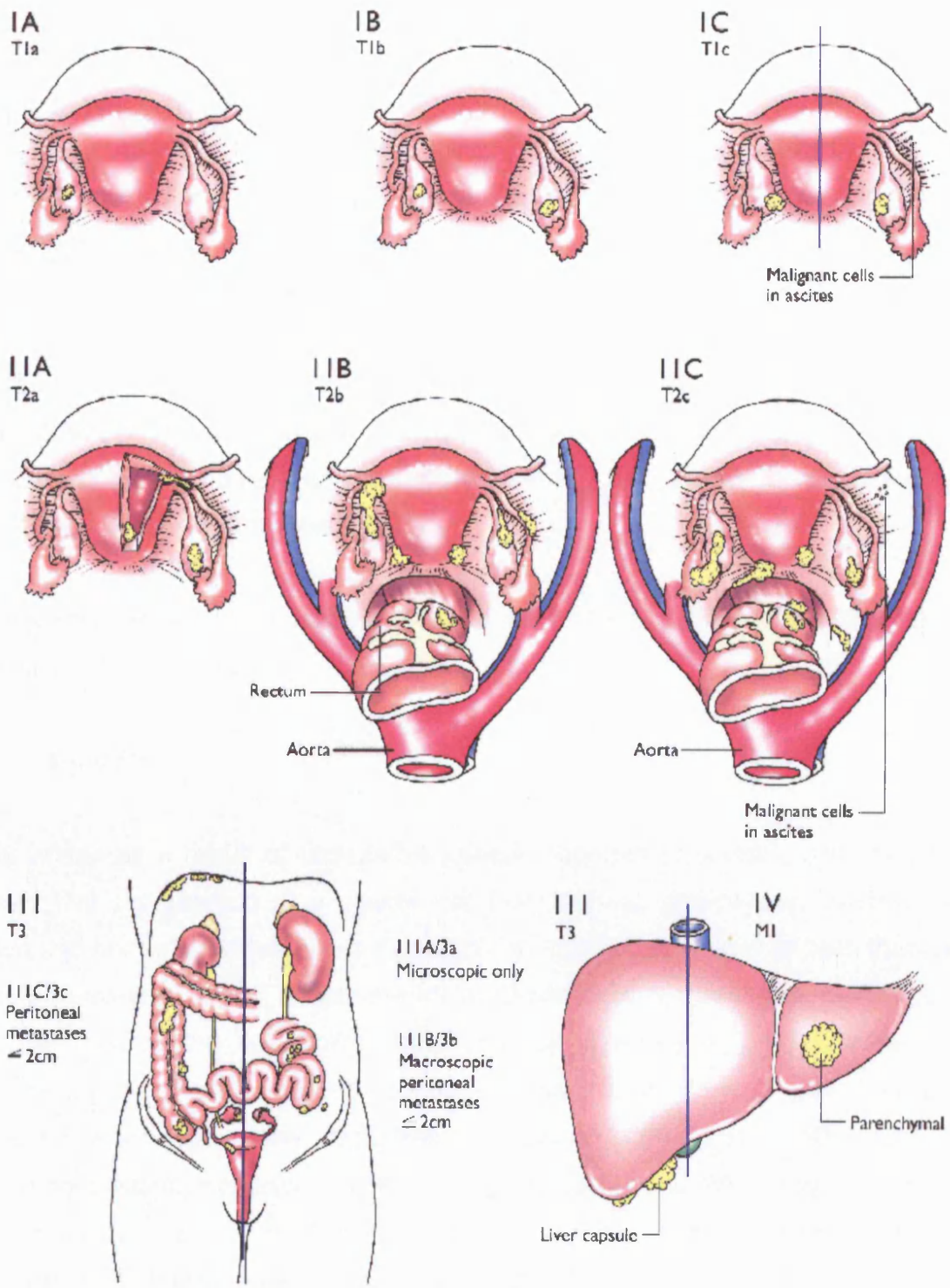


Figure 1.8. Staging of ovarian cancer: primary tumour and metastases (FIGO and TNM) (from FIGO, www.FIGO.org).

1.4 Molecular Biology of Sporadic Epithelial Ovarian Cancer

Epithelial ovarian cancer is thought to arise from the epithelial cells that line the surface of the ovary or from cells that line inclusion cysts immediately beneath the ovarian surface. By the time the disease is diagnosed, multiple tumour nodules are found studding the peritoneal surfaces. Molecular techniques have shown that more than 90% of EOC's are clonal diseases that originate from the progeny of single cells. Where ovarian tumours have been compared to their corresponding peritoneal metastases, both show inactivation of the same X chromosome, areas of loss of heterozygosity (LOH) on the same chromosomes, and, when present, have the same base mutated in the p53 tumour suppressor gene indicating monoclonality [Jacobs et al., 1992;Mok et al., 1992;Li et al., 1993].

Over the last decade a number of genetic abnormalities have been detected in epithelial ovarian cancers.

1.4.1 Introduction

Cancer arises as a result of cumulative genetic changes in somatic cells and their progeny. The progression of a cancer cell from normal, pre-cancer, cancer, local invasion and finally to metastasis is a function of a clonal expansion of cells that have acquired a selective growth advantage which allows them to outnumber neighbouring cells. The cells in the neoplastic clone undergo changes in gene activity as a consequence of genetic and epigenetic changes. Ultimately, a cell population establishes which can grow regardless of normal controls of proliferation and surrounding tissues. Hanahan and Weinberg [Hanahan and Weinberg, 2000] have identified six hallmark features which best define the cancer cell phenotype: self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. These traits are found in most and perhaps all types of human cancers.

The accumulation of genetic changes in cells during cancer development and progression have been most comprehensively described for colorectal cancer [Kinzler and Vogelstein, 1996] where the initial event is inactivation of the tumour suppressor gene APC, which allows a normal mucosal cell in the colonic epithelium to become an adenomatous polyp. Subsequently, mutations in oncogenes (e.g. C-myc or K-ras) or in tumour suppressor genes (e.g. p53 or DCC) lead to the progression from adenoma to carcinoma. Mismatch-repair genes recognise and correct mismatches which occur during DNA replication. In tumours where inactivation of the mismatch-repair genes has occurred, the rate at which DNA mutations occur is accelerated by the genetic instability. The APC gene has been described as the "gatekeeper" of the colonic epithelium, and in colon cancer, there is good evidence to suggest that disruption of the APC pathway is necessary for the initiation of the cancer. However, for epithelial ovarian cancer, as for most other cancers, such detailed knowledge of the early genetic events is lacking. The following details what is known about the molecular genetics of ovarian cancer.

1.4.2 Oncogenes and Tumour Suppressor Genes

Several factors are involved in the evolution of a cancer. These include alterations or mutations in specific oncogenes, and are divided into two categories: those that activate proto-oncogenes which promote cellular proliferation or inhibit cell death, and those that inactivate tumour suppressor genes which promote cell proliferation or promote cell death. These are discussed below.

1.4.2.1 Oncogenes

Oncogenes result from gain-of-function mutations in their normal cellular counterpart, proto-oncogenes, the normal function of which is to drive cellular proliferation. The most common mechanisms for mutational activation of proto-oncogenes are (i) gene amplification, leading to over-expression of an otherwise normal protein product, (ii) point mutation, leading to constitutive activation of a mutant form of the protein product and (iii) chromosomal translocation, which results in juxtaposition of the oncogene with the promoter region of a constitutively expressed gene, thereby

resulting in over-expression of the oncogene-encoded protein.

The Her-2/neu (c-erbB-2) is one of the most extensively investigated proto-oncogenes. It encodes a 185-kDa transmembrane glycoprotein with intrinsic tyrosine kinase activity, located on chromosome 17q21. It belongs to a family of growth factor receptors, including the epidermal growth factor receptor (EGFR or HER-1) and HER-3 and HER-4 [Carraway, III and Cantley, 1994]. HER-2 amplifies the signal provided by other HER receptors and so plays a central role in HER signalling. Over-expression of HER-2 oncogene has been found in 20-30% of ovarian cancers [Hellstrom et al., 2001;McKenzie et al., 1993;Berchuck, 1995] and is associated with a poor prognosis [Yu et al., 1993;McKenzie et al., 1993;Cirisano and Karlan, 1996;Rubin et al., 1993]. A poor prognosis could be related to a more aggressive phenotype due to ligand activation of the over-expressed receptor. Heregulin can inhibit clonogenic growth of ovarian cells that over-express HER-2 [Xu et al., 1999], but increases the ability of the same cells to invade matrigel membranes and to express proteases [Xu et al., 1997]. Therefore the poor prognosis associated with HER-2 over-expression may be due to increased invasiveness rather than to increased proliferation.

The G1 cyclin proteins regulate the progression of a cell through the G1-S phase of the cell cycle. The sequential activation of cyclin D1 followed by cyclin E leads to the inactivation of pRB1 (retinoblastoma protein) by phosphorylation, and allows the cell to enter the S phase of the cycle.

Up to 70% of ovarian tumours, mainly the late stage serous subtype over-express cyclin D1, and this confers a poor prognosis. The importance of cyclin E is less clear. 12-18% of ovarian tumours, especially clear cell tumours [Session et al., 1999] have been reported to over-express cyclin E, although no information was given regarding prognosis.

Myc is a member of the helix-loop-helix/leucine zipper superfamily of genes. It is located on chromosome 8q24 and encodes a DNA-binding protein which, on heterodimerisation with the MAX protein, binds to target DNA sequences and

induces transcription of several genes [Dang et al., 1999]. Myc plays a role in many cellular mechanisms including cell cycle control, differentiation, adhesion and apoptosis [Dang et al., 1999]. Studies have shown myc amplification in 28-50% of EOC's [Baker et al., 1990;Bauknecht et al., 1993;Katsaros et al., 1995]. The clinical relevance, is, however, not clear [Bauknecht et al., 1993].

Ras mutations occur in less than 20% of serous ovarian cancers [Enomoto et al., 1990]. However, physiologic activation of Ras has been found in a majority of ovarian cancer cell lines [Patton et al., 1998]. Activation of Ras may relate to the activity of receptor tyrosine kinases or to cross-talk from phosphatidylinositol 3-kinase (PI3-kinase).

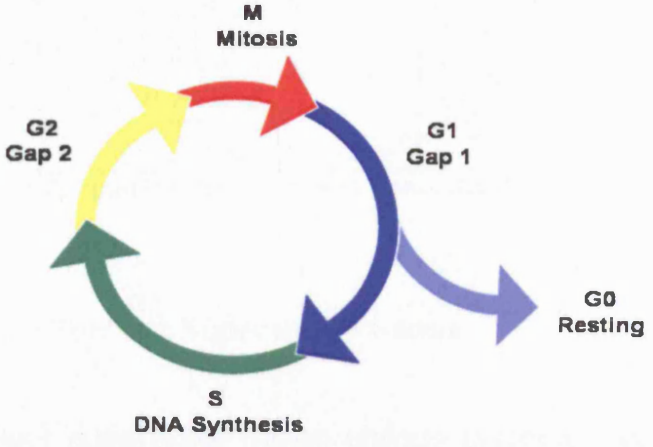


Figure 1.9. The phases of the cell cycle

The putative PIK3CA oncogene codes for the p110α catalytic subunit of PI3-kinase. It is located on chromosome 3q26; this region is increased in copy number in up to 40% of ovarian cancer [Iwabuchi et al., 1995]. Signalling by PI3-kinase has been shown to result in proliferation [Klippel et al., 1998], glucose transport and catabolism [Frevert and Kahn, 1997], cell adhesion [Khwaja et al., 1997], apoptosis [Kennedy et al., 1997], Ras signalling [Downward, 1998] and oncogenic transformation [Jimenez et al., 1998;Meili et al., 1998]. The increased copy number of PIK3CA is associated with increased PIK3CA transcription, p110α protein expression and PI3-kinase activity. As PI3-kinase signalling is associated with cancer-related functions, this implicates PIK3CA as an oncogene [Shayesteh et al., 1999]. In addition, treatment with PI3-kinase inhibitor decreases proliferation and increases apoptosis in ovarian

cancer tissue [Shayesteh et al., 1999].

In addition, downstream effectors of PI3-kinase, AKT1 and AKT2, have been found to be amplified in ovarian cancer. AKT2 is a gene on chromosome 19q which encodes a serine-threonine protein kinase. AKT2 has been shown to be amplified and over-expressed in two of eight cancer cell lines and two of 15 primary epithelial ovarian cancers [Cheng et al., 1992]. This study was confirmed by a larger series of 132 primary ovarian cancers where 14% had AKT2 amplification or over-expression. This subset consisted of high grade, late stage tumours which were associated with worse survival [Bellacosa et al., 1995].

Gene	Function	Approximate frequency
Her-2/neu	Tyrosine kinase	20%
Cyclin D1	Cell cycle	70%
Cyclin E	Cell cycle	12-18%
Myc	Transcription	28-50%
Ras	G protein	<20%
PIK3CA	Serine/threonine kinase	40%

Table 1.2. Putative Oncogenes in Epithelial Ovarian Cancer

1.4.2.2 Tumour Suppressor Genes

Tumour suppressor genes encode proteins that are normally involved in inhibiting proliferation, and inactivation of these genes plays a role in the development of most cancers. Knudson's "two-hit" model established the paradigm that both alleles must be inactivated in order to exert a phenotypic effect on tumorigenesis [Knudson, 1997]. The location and type of inactivating mutations may vary between cancers. Frequently, mutations in tumour suppressor genes alter the base sequence so that the encoded protein product is truncated because of the generation of a premature stop codon. Truncated protein products may result from several types of mutational events including nonsense mutations, in which a single base substitution changes a sequence from a specific amino acid to a stop codon (e.g., AAG to TAG). In addition, microdeletions or insertions of one or several nucleotides that disrupt the reading frame of the DNA (frameshifts) also lead to the generation of stop codons downstream in the gene. In some cases, missense mutations occur that change only a single amino acid in the encoded protein. A mutation in one allele, whether

germline or somatic, is revealed after somatic inactivation of the homologous wild-type allele. In theory, the same spectrum of mutational events could contribute to inactivation of the second allele, but what is typically observed in tumours is homozygosity or hemizygosity for the first mutation, indicating "loss" of the wild-type allele. The loss of the heterozygosity (LOH) has become recognised as the hallmark of tumour suppressor gene inactivation (see later).

The *p53* tumour suppressor gene maps to chromosome 17p13.1 and encodes a nuclear phosphoprotein. *P53* is considered the guardian of the genome because of its role in activating genes involved in growth arrest or apoptosis following DNA damage [Lane, 1992]. Alteration of the *p53* gene is the most common molecular event in ovarian cancer [Casey *et al.*, 1996;Hartmann *et al.*, 1994;Berchuck *et al.*, 1994]. In a normal cell, *p53* regulates transcription of other genes involved in cell cycle arrest, such as *p21*. *P53* is usually inactivated according to Knudson's "two-hit" model. This leads in the majority of cases to missense mutations that change a single amino acid in the DNA binding domain of the *p53* gene, and on to over-expression of non-functional p53 protein. The rate of *p53* mutation varies with the stage of disease; 10-20% of stage I/II cancers have the mutation, compared to 40-60% at advanced stages (III/IV). The increased rate of *p53* over-expression in late stage tumours may signify that this is a late event in ovarian carcinogenesis, and where *p53* is over-expressed it is associated with a worse prognosis [Marks *et al.*, 1991;Reles *et al.*, 2001]. However, the evidence is conflicting with some studies not confirming this result.

The RB1 (retinoblastoma) protein (pRb) acts as a critical cell cycle regulator. In its hypophosphorylated, active form, pRb binds to the E2F transcription factor and prevents the cell from entering S phase [Gillett and Barnes, 1998]. However, following phosphorylation by cyclin D1 and cyclin-dependent kinases (CDKs) and the subsequent release of E2F, the inhibitory effect of pRb is removed allowing progression from the G1 to S phase of the cell cycle. 30-52% of ovarian tumours demonstrate LOH at the RB1 locus, but express wild-type protein [Dodson *et al.*, 1994;Kim *et al.*, 1994;Li *et al.*, 1991]. These data suggest that either RB1 is inactivated without affecting protein levels, or that a second suppressor gene lies

close to the RB1 gene. A definitive role for RB1 in ovarian cancer remains to be determined.

The tumour suppressor gene CDKN2A, located on chromosome 9p21, encodes the cell cycle regulatory protein p16. Inactivation of the CDKN2A gene could lead to uncontrolled cell growth. Defects of the 'Rb/cyclin D1/p16 pathway' have been shown to play a critical role in the development of virtually all human malignancies. A significant positive association has been reported between p16 expression and clinical outcome for epithelial ovarian cancer patients. P16 acts by inhibiting binding between CDK4 and CDK6 and the D cyclins. This results in hypophosphorylation of pRb and causes cell cycle arrest at G1 phase. Homozygous deletion of p16 have been reported in 12-18% of ovarian tumours [Kudoh et al., 2002;Ichikawa et al., 1996]. There is evidence that p16 inactivation in primary serous papillary tumours occurs by methylation [Niederacher et al., 1999]. However, other studies have failed to find any methylation in these cancers [Ichikawa et al., 1996;Ryan et al., 1998]. These data suggest that the CDKN2A gene is involved in the tumorigenesis of ovarian cancer, but the exact mechanism of CDKN2A gene inactivation in serous papillary ovarian cancer remains unclear.

PTEN (phosphatase and tensin homolog deleted on chromosome ten), a tumour suppressor gene located on chromosome 10q23, is also known as MMAC1 (mutated in multiple advanced cancers) and TEPI (TGFß-regulated and epithelial cell-enriched phosphatase). PTEN encodes a protein that functions as a dual-specificity phosphatase in vitro [Myers et al., 1997], thereby recognising and dephosphorylating tyrosine and serine/threonine residues. PTEN is involved in the PI3-kinase-mediated growth signalling pathway [Maehama and Dixon, 1998], anoikis [Di Cristofano and Pandolfi, 2000], inhibition of cell adhesion, integrin-mediated cell migration and tumour invasion [Tamura et al., 1999a;Tamura et al., 1999b;Gu et al., 1999]. LOH at the PTEN locus has been reported in 28% of serous and 43% of endometrioid ovarian cancers [Obata and Hoshiai, 2000;Kurose et al., 2001]. These data suggest that PTEN may play a role in carcinogenesis of a subset of ovarian tumours.

The ARHI (ras homologue gene family, member I) gene, previously known as

NOEY2, has a 229-amino acid sequence and shares 54% amino acid homology with HRAS and 56 to 62% homology with ras-related proteins. PCR analysis of a genomic library and FISH [Yu et al., 1999] mapped the ARHI gene to 1p31, a region that is deleted in approximately 40% of breast [Loupart et al., 1995;Hoggard et al., 1995] and ovarian cancers [Yu et al., 1999] due to LOH.

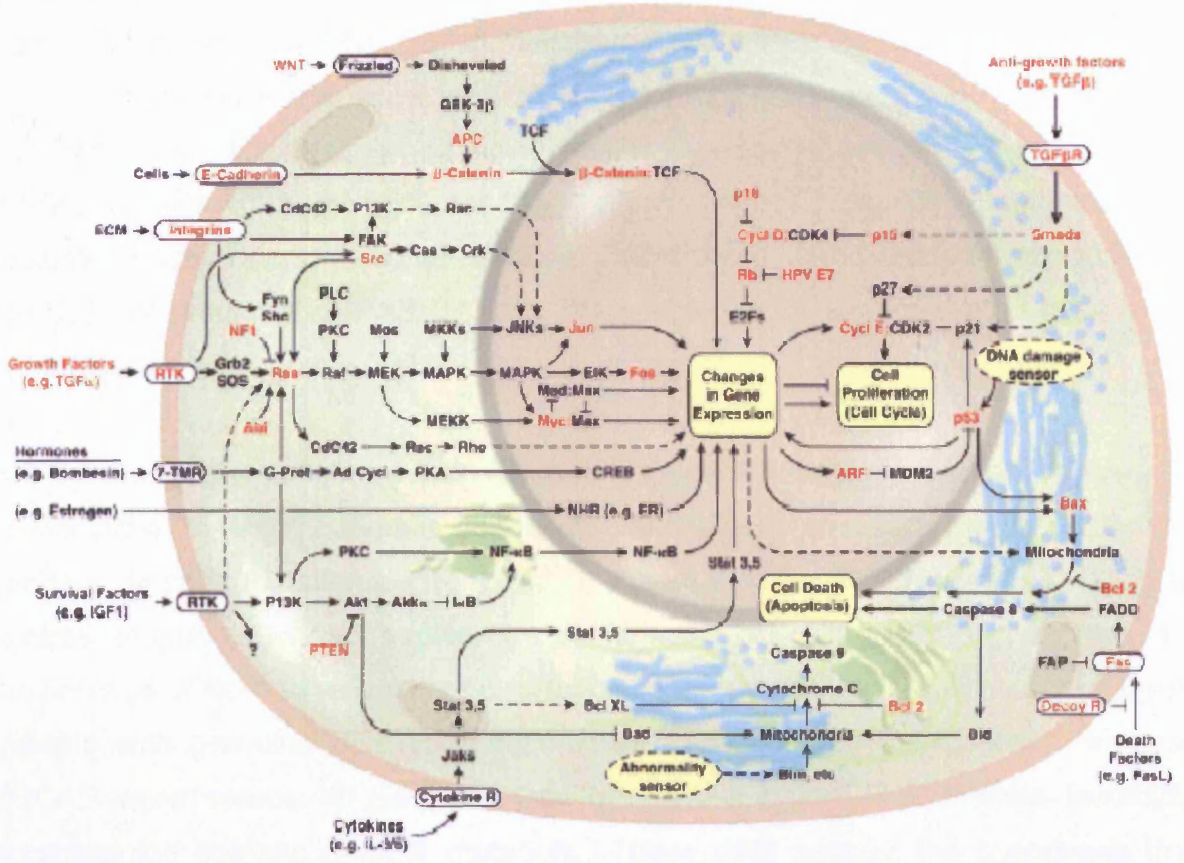


Figure 1.10. Role of oncogenes and tumour suppressor genes in signal transduction pathways and the cell cycle in tumour cells.

SPARC (secreted protein acidic and rich in cysteine) is an extracellular Ca(2+)-binding phosphorylated, acidic, matricellular glycoprotein of 43 kD, located on chromosome 5q31.3-q32 that associates with cell populations undergoing migration, morphogenesis, and differentiation. Studies on endothelial cells have established that its principal functions in vitro are counteradhesion and antiproliferation. The mechanism(s) underlying these antitumour effects is unknown. Brown et al demonstrated up-regulation of SPARC in reactive stroma associated with invasive ovarian cancer particularly at the tumour-stromal interface of the invading tumours, demonstrating the significance of the stroma to growth of cancers [Brown et al.,

1999]. Yiu and colleagues showed that SPARC expression in ovarian cancer cells is inversely correlated with the degree of malignancy. They treated human ovarian surface epithelial cells and ovarian cancer cells with SPARC, and revealed that SPARC inhibits the proliferation of both normal and cancer cells, but induces apoptosis only in cancer cells. This shows that down-regulation of SPARC is essential for ovarian carcinogenesis as cancer cells become sensitized to the apoptotic activity of SPARC during malignant transformation. This study showed the first direct evidence that putative SPARC receptors are present on human ovarian epithelial cells, with their levels being higher than in cancer cells. It is likely that binding of SPARC to its receptor triggers tissue-specific signalling pathways that mediate its tumour suppressing functions. Decrease in ligand-receptor interaction by the down-regulation of SPARC and/or its receptor is an essential step for ovarian carcinogenesis [Yiu et al., 2001].

BRCA1 mutations are the most common cause of hereditary ovarian cancers. A recent study, in which complete sequencing of the gene was performed 103 ovarian cancers, somatic mutations were found in at least 7 cases [Berchuck et al., 1998]. In contrast to women whose median age at diagnosis is typically in the mid-forties, the median age of women with somatic mutations was about 60 years. Similar to ovarian cancers with germline BRCA1 mutations, all of the ovarian cancers with somatic BRCA1 were serous. In addition, loss of the wild-type BRCA1 allele invariably accompanied somatic BRCA1 mutations. These data support the hypothesis that loss of BRCA1 function occurs by way of the classic tumour suppressor paradigm, with mutation of one copy and deletion of the other.

Gene	Chromosomal location	Function
P53	17p13	DNA stability Apoptosis
Rb1		Cell cycle regulator
CDKN2A	9p21	Cell cycle arrest
PTEN	10q23	Phosphatase
ARHI (NOEY2)	1p31	Induces p21 Inhibits cyclin D1
SPARC	5q31	Counteradhesion and antiproliferation
Doc2	5p13	Binds GRB2
LOT-1	6q25	Zinc finger
OVCA1	17p13	Unknown

Table 1.3.Putative Tumour Suppressor Genes in Epithelial Ovarian Cancer

1.4.3 Cytogenetic Alterations

There are genetic changes at the chromosomal as well as the molecular level. Comparative genomic hybridisation (CGH) studies have demonstrated that most ovarian cancers have chromosomal gains, losses and complex translocations often of large segments of chromosomes. Gains on chromosomes 3 and 8, and losses on chromosomes 16 and 17 are the most common karyotypic findings in ovarian cancer [Iwabuchi et al., 1995]. Chromosome regions frequently affected with allelic imbalance include 1p [Chenevix-Trench et al., 1992], 2q [Saretzki et al., 1997], 3p [Lounis et al., 1998], 5q [Tavassoli et al., 1996], 6q25 [Colitti et al., 1998], 7q31 [Zenklusen et al., 1995], 9p [Devlin et al., 1996], 11p15 [Lu et al., 1997], 11q [Launonen et al., 1998], 12p12 [Hatta et al., 1997], 13q [Yang-Feng et al., 1992], 17q12 [Foulkes et al., 1993], 18q [Saretzki et al., 1997], 19q [Bicher et al., 1997], 22q [Englefield et al., 1994], and Xq [Choi et al., 1997]. There are differences between serous adenocarcinomas and the other histological subtypes. Serous tumours mostly have gains at 1q and 11q, whereas endometrioid tumours have gains at 1q and 10q, and mucinous have gains at 17q. Once the areas of chromosomal abnormality are pinpointed, molecular techniques such as LOH studies using microsatellite markers can be applied to narrow the target to the gene-locus level. These regions of recurrent abnormality are thought to encode genes involved in ovarian carcinogenesis when differentially expressed as a result of abnormal copy number or mutation. Certain oncogenes and tumour suppressor genes have been identified in several regions of allelic imbalance, and specific karyotypic abnormalities have been associated with clinical outcome [Saretzki et al., 1997;Launonen et al., 1998;Schwab and Amler, 1990], although it is unclear whether the extent of these genetic alterations reflects the inactivation of multiple tumour suppressor genes or whether it is the result of generalised instability of the genome. Many studies have consistently shown that poorly differentiated, late stage tumours have more genetic alterations than well differentiated, early stage or LMP tumours [Iwabuchi et al., 1995;Dodson et al., 1993]. This could be due to an accumulation of genetic changes during the evolution of a tumour, or it could be that late stage, high-grade cancers are more aggressive even early in their development due to specific mutations or increased genomic instability. If the latter theory is correct, then this could have significant

implications for screening, as cancers which are inherently more aggressive are more likely to metastasise confer a worse prognosis.

1.4.4 Peptide Growth Factors

Growth factors are produced by many different cell types and exert their effects via autocrine and paracrine mechanisms. They function as stimulators or inhibitors of the division, differentiation and migration of cells and are involved in carcinogenesis, in which they influence a variety of functions including cell proliferation, cell invasion, metastasis formation, angiogenesis, local immune system functions and extracellular matrix synthesis.

The EGF-related peptides have been the most extensively studied in ovarian cancer. EGF, transforming growth factor (TGF)-α and amphiregulin bind to, and activate, the EGF receptor (EGFR). All three factors have been identified in ovarian tumours and cultured ovarian cancer cells. Studies have shown TGF-α to be present in 50-100%, EGF in 28-71% and amphiregulin in 18% of ovarian cancers [Kommoss et al., 1990;Morishige et al., 1991;Owens et al., 1991b;Kohler et al., 1992;Stromberg et al., 1994]. TGF-α has been detected in the sera of 62% of women with ovarian cancers compared with 28% with benign ovarian tumours and 11% of normal female controls [Chien et al., 1997]. Similarly, TGF-α has been detected in the urine of 79% of ovarian cancer patients compared with 17% of patients with benign tumours and 23% of controls [Feldkamper et al., 1994].

The EGFR, a glycosylated membrane-spanning protein, has been found to be present in 33-75% of primary ovarian tumours by ligand binding [Bauknecht et al., 1988;Battaglia et al., 1989;Morishige et al., 1991;Owens et al., 1991a] and by immunohistochemistry [Berchuck et al., 1991;Henzen-Logmans et al., 1992;Owens et al., 1992]. Levels of EGFR are higher in malignant than benign tumours or normal ovary, suggesting a possible role in malignant progression, and are related to poor prognosis [Bauknecht et al., 1988;Battaglia et al., 1989;Berchuck et al., 1991].

The TGF-β family are involved in cell growth regulation, angiogenesis and tissue

remodelling. TGF-β peptides have an inhibitory rather than stimulatory role. They have been shown to inhibit the growth of both ovarian cancer cells (by 95%), immortalised ovarian cancer cell lines [Berchuck et al., 1990;Marth et al., 1992;Bartlett et al., 1992;Jozan et al., 1992] and normal ovarian epithelial cells grown in culture [Hurteau et al., 1994]. It has been suggested that TGF-β is an important regulator of normal ovarian epithelium and autocrine inhibition may be lost in many cancer cell lines. Some ovarian cancers that are growth-inhibited by TGF-β are also more likely to undergo apoptosis [Havrilesky et al., 1995].

Inhibin, another member of the TGF- β family, is increased in the sera of patients with EOC and is associated with increased survival [Blaakaer et al., 1993;Cooke et al., 1995].

The insulin-like growth factors (IGF), IGF-I and IGF-II are important mitogenic growth factors which show close structural homology to insulin [Barreca and Minuto, 1989]. The IGFs, IGF receptors (insulin, type I and type II receptors) and IGF binding proteins (IGFBPs) have been identified in many ovarian tumours [Beck et al., 1994;van Dam et al., 1994;Weigang et al., 1994] and ovarian cancer cell lines [Resnicoff et al., 1993;Hofmann et al., 1994] and have a stimulatory effect. As both IGF-I and its receptor are co-expressed, and autocrine mechanism can be postulated. IGFBP-2 levels have been shown to be high in sera of ovarian cancer patients [Flyvbjerg et al., 1997] and in malignant ovarian cyst fluid [Karasik et al., 1994] and correlate with aggressiveness of the tumour.

Platelet-derived growth factor (PDGF) expression is elevated in ovarian cancers and undetectable in normal ovaries and benign tumours [Henriksen et al., 1993;Versnel et al., 1994]. Tumours expressing the PDGF receptor are associated with shorter overall survival [Henriksen et al., 1993]. The concomitant expression of both PDGF and its receptor is related to disease progression, suggesting autocrine growth regulation.

Basic fibroblast growth factor (bFGF) and its receptor are both expressed in ovarian cancer cells, again suggesting an autocrine mechanism, and function in a stimulatory

manner [Crickard et al., 1994]. FGFs stimulate both mitogenesis and angiogenesis.

In summary, ovarian cancers produce and respond to many peptide growth factors such as epidermal growth factor, transforming growth factor-α, insulin-like growth factors, platelet-derived growth factor and basic fibroblast growth factor. The concomitant over-expression of both growth factor and receptor suggests an autocrine stimulatory mechanism. However, as normal ovarian epithelial cells also produce and respond to the same peptide growth factors found in ovarian cancer cells, it is unclear whether these growth factors play a role in the development of tumours or merely maintain cell growth once tumours are established.

1.4.5 Metastasis Suppressor Genes

1.4.5.1 E-Cadherin (CDH1)

E-cadherin is a transmembrane glycoprotein, which, through its attachment to the actin cytoskeleton via α, ß and γ-catenin, functions as a cell adhesion molecule. Loss of functional E-cadherin or catenin is associated with ability of cancer cells to detach from the primary tumour, thereby facilitating metastasis progression. E-cadherin (CDH1) mutations are rare in ovarian cancer [Risinger et al., 1994]. CDH1 protein expression is reduced in poorly differentiated ovarian tumours and ovarian cancer metastases compared with primary tumours, implying that loss of CDH1 correlates with acquired invasive and metastatic potential [Davies et al., 1998]. The CDH1 pathway may potentially be disrupted via mutations in the catenin genes, and missense mutations of ß-catenin have been reported in endometrioid ovarian tumours but rarely in other histological subtypes [Gamallo et al., 1999;Wright et al., 1999]. Reduction or absence of either ß- or α-catenin expression in ovarian tumours is associated with increased metastatic potential and poor prognosis [Davies et al., 1998].

1.4.5.2 Nm23 (NME1 & 2)

The nm23-H1 (NME1) and nm23-H2 (NME2) genes are located on chromosome

17q21.3 and encode nucleoside diphosphate kinase A and B respectively. These proteins catalyse the phosphorylation of nucleoside diphosphates using ATP, resulting in the production of the corresponding nucleoside triphosphates [Lombardi et al., 2000]. Reduced expression of NME1 and NME2 is associated with a highly metastatic phenotype in ovarian tumours and cell lines [Simone et al., 2001;Tas et al., 2002;Galani et al., 2002]. NME1 may also be important in the progression of ovarian tumours as reduced NME1 expression is associated with late stage disease lymph node involvement and distant metastasis [Mandai et al., 1994].

1.4.6 Cell Survival and Cell Death Pathways

1.4.6.1 Senescence

Normal somatic cells can undergo division only a finite number of times, and after a certain number of doublings, undergo senescence. This is characterised by arrest of proliferation without loss of biochemical function or viability. The maximum number of doublings has often been referred to as the "Hayflick limit", that is, there is a counting mechanism or "molecular clock" which can trigger senescence at the appropriate time. Cellular senescence is due to the shortening of repetitive DNA sequences (TTAGGG) called telomeres that cap the ends of each chromosome. Telomeres stabilise chromosomes and prevent recombination during mitosis. Chromosomes have long telomeric sequences at birth, which then become progressively shorter each time the cell divides. Cancer cells must bypass senescence in order to gain the ability to replicate an unlimited number of times, and undergo immortalisation, and essential step in malignant transformation of normal cells. They accomplish this by inducing the expression of telomerase which acts to lengthen the telomeres [Shay, 1998]. Telomerase is a ribonucleoprotein whose RNA acts as a template for telomere extension and the protein catalyses the synthesis of new telomeric repeats.

The expression of telomerase is limited to those cells actively dividing, so it has been suggested that telomerase would be a useful diagnostic marker in patients with cancer. Researchers have demonstrated that telomerase activity is detectable in most ovarian cancers [Duggan et al., 1998;Wan et al., 1997]. It has been suggested

that persistence of telomerase activity in peritoneal washings after primary therapy for advanced ovarian cancer may predict the presence of microscopic residual disease in some cases even when the cytological washings and biopsies are negative. More studies are required before this is demonstrated as a feasible clinical tool.

1.4.6.2 Apoptosis

Apoptosis plays a vital role in a variety of human diseases, including cancer. It is an active, energy-dependent process that involves endonuclease and protease cleavage of DNA and proteins. Morphologically it is characterised by cytoplasmic shrinkage, active membrane blebbing, chromatin condensation and fragmentation into membrane-enclosed vesicles [Kerr et al., 1972;Wyllie et al., 1980]. This is different to necrosis which is characterised by loss of osmoregulation and cellular fragmentation. Because the balance between cell proliferation and cell death controls the size of a cell population, tumour growth can result from either increased proliferation or decreased apoptosis. Suppression of apoptosis can lead to cells becoming malignant by allowing the accumulation of genetic mutations, growth factor-independent tumour cell growth, and escape from cell-cycle checkpoints which would normally induce apoptosis.

An important role of apoptosis is to eliminate cells which have undergone mutation, thereby preventing malignant transformation. When cells are exposed to mutagens such as radiation, the cell cycle is arrested so that DNA repair may take place. If the DNA cannot be repaired, then apoptosis has to occur to remove any abnormal cells before they become malignant. The p53 tumour suppressor gene plays a critical part in regulation of cell cycle arrest and apoptosis. Other genes involved in this process include bcl-2, which was first identified at a translocation breakpoint in B-cell lymphomas. Bcl-2 expression inhibits apoptosis although paradoxically, the continued expression of bcl-2 in ovarian cancers is associated with a good prognosis [Herod et al., 1996;Henriksen et al., 1995]. The bcl-XL expression (a structural and functional homologue of bcl-2) also inhibits apoptosis in ovarian cancer cells in response to chemotherapy [Liu et al., 1998]. Other related genes such as bax and

bcl-XS are pro-apoptotic. Studies have found high bax expression in primary ovarian cancers, and this was associated with a good response to chemotherapy [Tai et al., 1998].

1.4.6.3 Proliferation

Increased proliferation is a hallmark of cancer cells. Proliferation can be measured by assessing the DNA content of cells or by immunohistochemical staining Ki67 or proliferative cell nuclear antigen (PCNA), which are proteins expressed by proliferating cells. Ki67 is expressed in G1, S and G2 phases of the cell cycle but not in G0 cells. Ki67 correlates with poor survival in ovarian cancer [Anttila et al., 1998].

1.4.6.4 DNA Index

Aneuploidy is present in most malignant tumours and in many early stage carcinomas. It is defined as cells with chromosome numbers greater or smaller than the normal diploid complement. A variety of methods have been used to demonstrate aneuploidy including karyotyping [Wang *et al.*, 2000], flow cytometry [Eissa *et al.*, 1998], image analysis [Guidozzi *et al.*, 1996] and fluorescence *in situ* hybridisation (FISH) [Mark *et al.*, 1999]. Many groups have investigated the value of ploidy in ovarian cancer. Advanced stage (III/IV), poorly differentiated tumours are aneuploid in 65-90% of cases and aneuploid tumours have an aggressive clinical course and a poor outcome [Ozalp *et al.*, 2001;But and Gorisek, 2000]. Aneuploidy is present early during tumour progression [Skirnisdottir *et al.*, 2001] and it appears that the altered DNA content, and its underlying cause, may play a role in both the development and progression of ovarian tumours. Therefore although the DNA index may not be predictive in patients with advanced stage ovarian carcinoma, it may help predict the risk of recurrence in patients with early-stage disease as these tumours are less commonly aneuploid [Schueler *et al.*, 1996].

1.5 Invasion and Metastasis of Ovarian Cancer

The spread of cancer, either by local invasion or metastasis through the bloodstream or lymphatics is the hallmark of malignancy [Liotta, 1992]. Cells leave the primary tumour and enter either the lymphatic or vascular network before spreading to other organs. Ovarian cancer spread occurs at two levels: (i) in the abdominal cavity and (ii) in the retroperitoneal space. The first method, spread into the abdominal cavity involves individual cancer cells shedding from the ovary and entering the peritoneal fluid. These carcinoma cells then attach to the layer of mesothelial cells that line the inner surface of the peritoneal cavity. Once ovarian carcinoma cells adhere to mesothelial cells, they migrate through the layer of mesothelial cells, invade the local organs and can spread to distant sites. This process of cancer cell adhesion, migration and invasion can eventually lead to death of the patient. The second method, spread into the retroperitoneal space, involves spread through lymphatic channels leaving from the hilus of the ovary and on to inguinal, iliac, obturator and para-aortic lymph nodes. Spread to lymph nodes is not a late event as in apparently stage I tumours, nodal metastases have been reported in between 14 to 24% of cases [Burghardt et al., 1991]. The incidence of nodal metastasis increases with stage, involving 50% of stage II and 70% of stage III tumours. Ascites often co-exists with ovarian cancer at the time of presentation. Peritoneal fluid is constantly formed as a transudate from small blood vessels, and is resorbed by subdiaphragmatic lymphatics. Ascites develops when the balance of equilibrium is towards formation over resorption. In ovarian cancer patients, ascites forms when lymphatic channels are blocked by tumour, or due to increased vascular permeability.

At the molecular level, cells of the primary tumour acquire genetic mutations which confer the ability to survive. A cell population evolves that disregards the normal controls of proliferation and territory. Hanahan and Weinberg [Hanahan and Weinberg, 2000] described "six hallmarks of cancer": disregard of signals to stop proliferating and of signals to differentiate; capacity for sustained proliferation; evasion of apoptosis; invasion and angiogenesis (Figure 1.11). The primary tumour cannot grow beyond 1-2mm in size without acquiring a blood supply (the "angiogenic switch") [Folkman, 1971]. Angiogenesis is the growth of new capillary blood vessels,

and important mediators of this process are vascular endothelial growth factor (VEGF) and its mitogenic receptor VEGFR-2, which is localised on endothelial cells, and basic fibroblast growth factor (bFGF), produced by both the tumour and by infiltrating cells. High VEGF expression in carcinoma cells is an independent prognostic factor for poor prognosis in both advanced [Hartenbach et al., 1997] and early stage [Paley et al., 1997] disease. Angiogenesis is also assisted by extracellular matrix (ECM) breakdown and it appears that matrix metalloproteinases (MMPs) are expressed early in tumour growth. The neovasculature of the tumour is more permeable than normal vessels due to fenestrated basement membranes and fewer cell junctions, which assist the metastatic process.

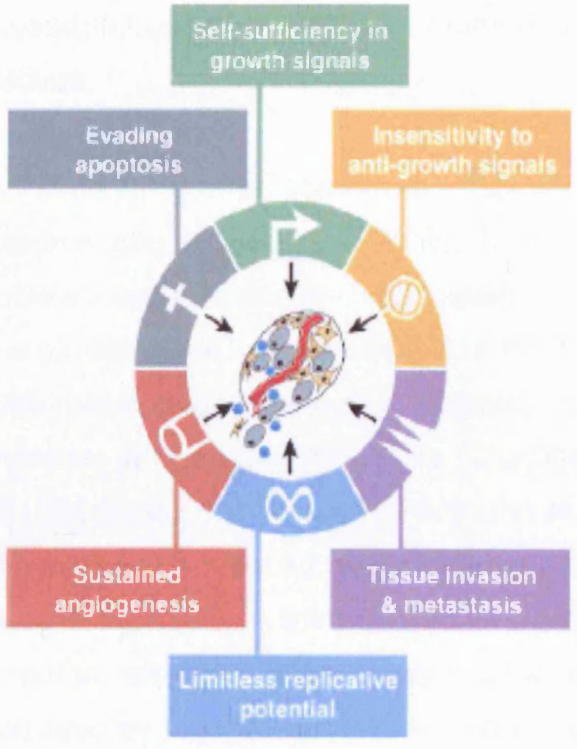


Figure 1.11. The six hallmarks of cancer.
Figure taken from Hanahan & Weinberg, (2000).

The next stage of the metastatic process involves movement through the tumour basement membrane and breakdown of the ECM, which is composed of collagens, fibronectin, glycosaminoglycans and laminins. The ECM provides structural support for cells, ligands for cellular receptors such as integrins and produces inactive forms of growth factors and proteases. Of these proteases, MMPs are produced by both

stromal and endothelial cells in response to factors released by tumour cells, but they are also produced by tumour cells themselves. They are regulated in balance with tissue inhibitors of metalloproteinases (TIMPs). Two metalloproteinases, MMP-2 and MMP-9 (72kDa and 92kDa type IV collagenases or gelatinase A and B, respectively), play a role in ovarian cancer. They are able to degrade type IV collagen, which is a major component of the basement membrane [Nelson et al., 2000]. Secretion of MMP-2 and MMP-9 has been observed in several ovarian cancer cell lines and detected in ascitic fluid from patients with advanced ovarian cancer. The invasiveness of ovarian cancer cell lines correlates with MMP-2 and MMP-9 expression in vitro [Moser et al., 1994;Young et al., 1996;Afzal et al., 1996]. MMPs probably act in two ways in ovarian cancer; they enable ovarian cancer cells to detach from the epithelial surface and migrate into the peritoneal cavity and also to invade through the basement membrane into the ovarian stroma, then to adjacent tissues.

A second group comprises the serine proteases including urokinase-type plasminogen activator (u-PA) which must bind to its membrane receptor (uPAR) to facilitate cell invasion and metastasis. The enzymatic activity of uPA is regulated by the plasminogen activator-inhibitors PAI1 and PAI2. High levels of uPAI1 (urokinase-type plasminogen activator inhibitor) in ascitic fluid of ovarian cancer patients correlate with a good prognosis [Chambers et al., 1995], but high uPA [Konecny et al., 2001] and PAI1 and/or uPA [Kuhn et al., 1999] expression correlate with a poor prognosis in advanced stage ovarian cancer in some, but not all studies [van der Burg et al., 1996]. A third degradative protein is heparanase, an endoglycosidase for heparan sulphate (HS) in glycosaminoglycans, which regulates bFGF and Pas released by HS. The proteases and heparanase act in concert to degrade the ECM and enable breakthrough of ovarian cancer cells from the tumour bulk, and also facilitate the release of cytokines and growth factors that induce proliferation and migration of endothelial, mesenchymal and tumour cells. The local stromal environment plays a pivotal role in ovarian tumorigenesis, with interactions between the different cell types within the tumour and the microenvironment paralleling neoplastic change (Figure 1.12).

Invasion is characterised by cellular movement. Ovarian cancer cells move either by passive translocation in response to mechanical pressure or by active migration. In many ovarian epithelial tumours, E-cadherin (the adherens junction component) is lost, leading to reduced cell-cell adhesion, enabling cells to detach and spread to the peritoneum [Fujioka et al., 2001]. Low E-cadherin levels are strong markers of poor prognosis [Herrera et al., 2002].

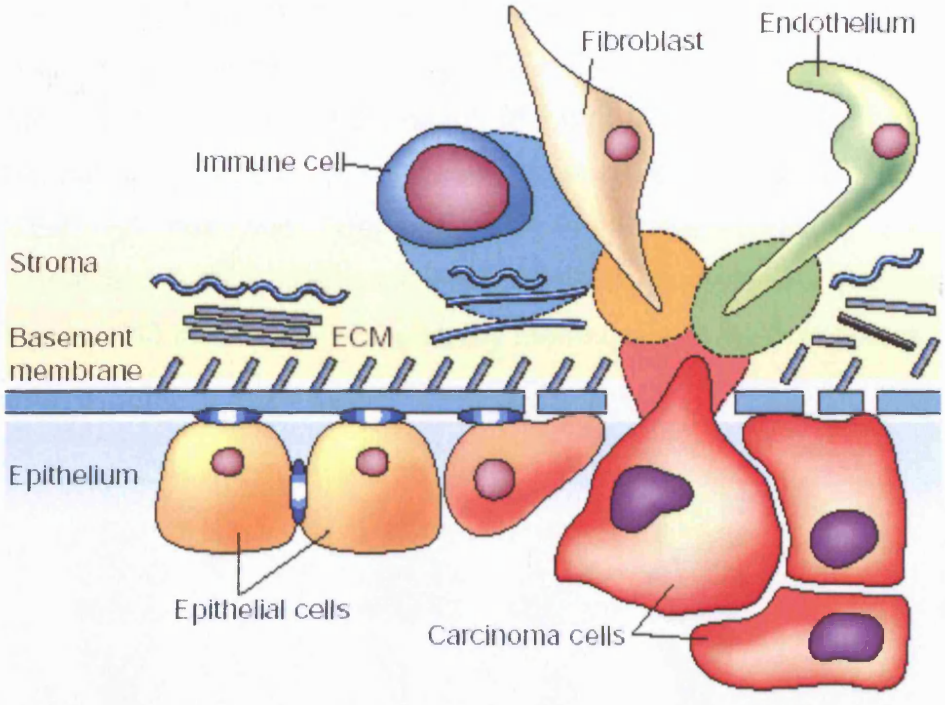


Figure 1.12. The interplay between epithelial tumour cells and the stroma.
Figure taken from Liotta & Kohn, (2001).

The next stage of dissemination of malignant cells from the primary tumour to adjacent tissue or to distant organs via lymphatics or the bloodstream is the hallmark of metastasis. Metastasis is the leading cause of death in patients in most epithelial malignancies. Haematogenous spread regulated by VEGF controlling angiogenesis is well characterised. However, this is not the main route of spread for ovarian cancer, and it is possible that lymphangiogenesis plays an even more important role. VEGF is known to signal through two tyrosine kinase receptors, VEGFR-1 and VEGFR-2, which are expressed primarily but not exclusively on vascular endothelial cells. The field of lymphangiogenesis research gained momentum with the discovery of a third VEGF receptor, VEGFR-3, which was found to be predominantly expressed

on lymphatic vessels during development [Kaipainen et al., 1995]. VEGF was not found to bind to VEGFR-3, but instead two novel ligands, VEGF-C and VEGF-D were found to bind VEGFR-3 [Joukov et al., 1996;Achen et al., 1998]. Researchers have shown that VEGFR-3 and its ligands VEGF-C and VEGF-D have an important role in tumour-induced lymphangiogenesis and lymphatic metastasis in mouse models [Makinen et al., 2001;Stacker et al., 2001]. VEGF-C gene expression in ovarian cancer cell lines has been directly correlated with invasion [Ueda et al., 2001], suggesting cancers expressing VEGF-C have greater metastatic potential due to their ability to stimulate formation of lymphatic endothelia which leads to lymphatic tumour cell spread. In addition, increased expression of VEGF-C, VEGF-D and VEGFR-3 has been demonstrated in ovarian cancers, and was significantly associated with lymph node and peritoneal metastases [Yokoyama et al., 2003]. Figure 1.13 shows the VEGF family members and their receptors.

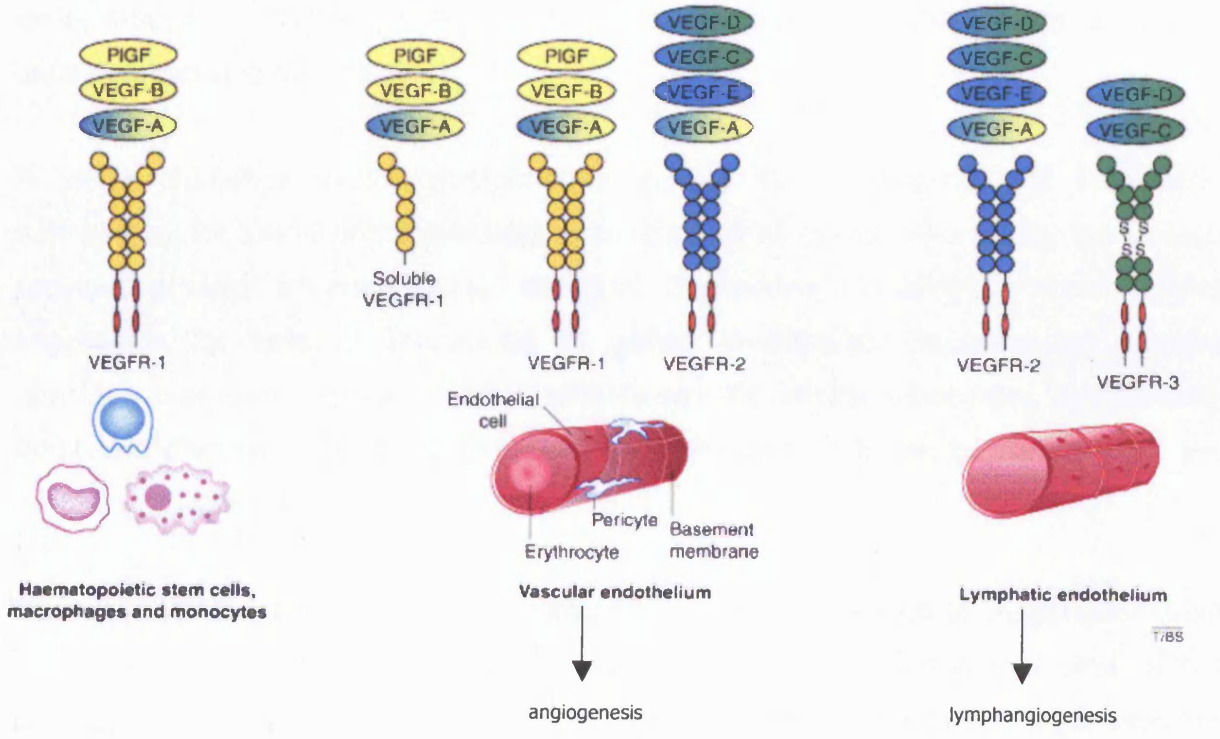


Figure 1.13. Members and receptors of the VEGF family.
Adapted from Cross et al, (2003).

1.6 Microarrays

1.6.1 Introduction

The completion of the human genome project will vastly increase our knowledge of the genomic sequences of humans and the genes that they encode. Traditional techniques for detecting and quantitating gene expression levels include northern blots [Alwine et al., 1977], S1 nuclease protection [Berk and Sharp, 1977], differential display [Liang and Pardee, 1992], sequencing of cDNA libraries [Adams et al., 1991;Okubo et al., 1992] and serial analysis of gene expression (SAGE) [Velculescu et al., 1995]. These are both laborious and time consuming. With the advent of microarray technology, the study of gene function has been revolutionised. The expression pattern of thousands of genes can be screened in parallel to give a gene-expression or molecular profile of a certain cell or tissue. Microarray technology has been advancing rapidly in the past few years, and improvements have been made at every step, including array production, probe manufacture, hybridisation, scanning, data analysis and data mining.

A major challenge in the postgenomic age is the development of systematic approaches for identifying the biological function of all genes. Microarray technology provides a major advance toward this goal. It provides a strategy to monitor gene expression for tens of thousands of genes in parallel. Because the relative abundance of gene expression often reflects specific cellular processes, a sufficiently large and diverse set of gene expression profiles from different tissue samples are providing insight into gene functions.

Two main DNA microarray technologies have been developed to investigate gene expression analysis. These are oligonucleotide-based microarrays and cDNA microarrays. These are discussed and compared with emphasis on oligonucleotide arrays.

1.6.2 Comparison Between Oligonucleotide and cDNA Arrays

The basic principle of both types of microarrays is the precise positioning of DNA fragments (probes) at high density on a solid support so that they can function as molecular detectors. The probe refers to the DNA arrayed since it is equivalent to the probe used in a northern blot analysis. Every strand of nucleic acid has the capacity to recognise complementary sequences through base pairing. Nucleic acid arrays work on the principle that labelled RNA or DNA molecules in solution (target) hybridise to DNA molecules attached at specific locations on a surface. This process of hybridisation can be highly parallel; every RNA/DNA sequence can be investigated simultaneously.

1.6.2.1 Probes

Probes for cDNA arrays are usually PCR products representing specific genes, with sequences generated from databases such as GenBank or UniGene, and partially sequenced cDNAs (or ESTs) from any cDNA library of interest. The probes are robotically printed on to glass slides or nylon membranes as spots at defined locations, and are referred to as probe cells. The spots are 100-300μm in size, are spaced the same distance apart and contain one single length (up to 1,000 base pairs) of double-stranded DNA. Using this technique, glass substrates the size of a microscope slide can accommodate approximately 30,000 cDNAs.

Oligonucleotide arrays differ from spotted cDNA arrays in that they utilise short 20-25-mer oligonucleotide lengths based on DNA sequences generated from a given gene. These can either be synthesised in situ directly on to silicon wafers by photolithography (high-density oligonucleotide arrays from Affymetrix, http://www.affymetrix.com) or presynthesised then printed on to glass slides. In the case of photolithographically synthesised arrays, ~107 copies of each selected oligonucleotide are synthesised base by base in hundreds of thousands of different 24μm x 24μm areas on a 1.28 x 1.28 cm glass surface. Because oligonucleotide arrays are designed and synthesised based only on information of the DNA sequence, physical intermediates such as PCR products and cDNAs are not needed.

The 25mer oligonucleotides are designed so that they are as unique, sensitive and sequence-specific as possible, and as dissimilar to other gene family members, other genes and other RNAs which may be present in the sample (e.g. rRNAs, tRNAs and mRNA). The arrays are designed in silico (designed by computer programs that select the best probes without cross hybridisation to other targets) negating the need to prepare, verify, quantitate and catalogue a long list of PCR products and cDNAs. In addition, because probes can be designed to represent the most unique part of a particular transcript, it is possible to detect closely related genes and splice variants. For both types of array, the probes are designed from sequences near the 3' end of the gene (near the poly-A tail in eukaryotic mRNA) in order to decrease problems with RNA degradation.

An important feature of oligonucleotide arrays is probe redundancy, where the same gene has many oligonucleotides with different sequences designed to hybridise to it. This greatly improves the signal-to-noise ratio, improves the accuracy of RNA quantitation, prevents effects due to cross-hybridisation and greatly reduces the false positive and miscall rates. Another level of probe redundancy is the use of mismatch (MM) control probes which are identical to the perfect match (PM) probes except for one base pair. This increases specificity by removing background and cross-hybridisation signals, and enables the array to discriminate between "real" signals and those due to non-specific or semi-specific hybridisation. Redundant PM/MM probe sets can differentiate whether the intended RNA molecule is generating a hybridisation signal; a single spot intensity generated by cDNA arrays is not as discriminative.

1.6.2.2 Target Preparation

Oligonucleotide arrays differ to cDNA arrays in the preparation of the target (sample of interest). For both types, RNA extracted from cells or tissue, transcribed into DNA, labelled, hybridised to the DNA probes on the microarray surface then detected by either phosphor-imaging or fluorescence scanning. Oligonucleotide arrays use double-stranded cDNA to serve as template for a reverse transcription reaction in which labelled nucleotides are incorporated into cRNA. The most commonly used

label is nonfluorescent biotin which is subsequently labelled by staining with a fluorescent streptavidin conjugate. These arrays are very robust and produce highly reproducible data, so that signals from separate arrays can be compared with one another. In the case of cDNA spotted arrays, however, the process of gridding is not accurate enough to allow comparison between separate arrays. Therefore the target preparation process involves labelling of two distinct RNA populations by two separate fluorescent dyes (the most commonly used are Cy3 and Cy5), mixing and hybridisation to the same array.

1.6.2.3 Scanning

After the targets are labelled and hybridised (usually for approximately 16 hours), the arrays are scanned. For the oligonucleotide arrays, the light emitted from each probe cell is proportional to the bound target at each location, therefore the presence or absence of a particular transcript above background and noise levels can be assessed. In the case of cDNA arrays, two fluorescent dyes are used, causing competitive binding, so the ratio of fluorescence intensities between the two dyes in one spot gives information on the extent of up- or down-regulation of a gene.

1.6.3 Data Analysis

Microarray experiments generate vast amounts of data. This is usually in the form of long lists of spot intensities and intensity ratios, generated either by comparing several samples to a control (oligonucleotide arrays) or by pairwise comparison of two samples (cDNA arrays). The aim is then to convert these data into meaningful information.

1.6.3.1 Normalisation

The intensity information from the values of each of the probes in a probeset is combined together to get an expression measure (MAS 5.0 statistical algorithm from Affymetrix 2001). Normalisation is needed when dealing with experiments from more than one array. There are two main types of variation, interesting variation and

obscuring variation. An example of interesting variation is the differences in gene expression between normal and malignant tissues. Obscuring variation is that introduced by performing the experiments themselves e.g. unequal quantities of starting RNA, differences in sample preparation (e.g. labelling) and array processing (scanning) and differences in hybridisation efficiency.

Affymetrix normalisation is performed on expression summary values. This approach does not work well in the case of non-linear relationships between arrays. Other approaches include using non-linear smooth curves [Li and Hung, 2001] and transforming the data so that the distribution of probe intensities is the same across a set of arrays; both parametric and non-parametric methods can be used to achieve this. These approaches depend on the choice of a baseline array.

1.6.3.2 Clustering

Analysing microarray data involves classifying individual genes into a set of categories. Some methods classify genes into predefined categories (supervised clustering), whereas other methods use categories that are discovered during the analysis (unsupervised clustering). Both supervised and unsupervised clustering methods assign a category to gene (using expression measurements across array experiments), the categories being biological functions of genes or diagnoses associated with pathologic specimens.

1.6.3.2.1 Unsupervised Clustering

Unsupervised clustering techniques for analyzing microarrays include hierarchical clustering, k-means clustering and self-organising maps. They are useful for initial data exploration, and have been validated under certain circumstances by their successful "rediscovery" of known classes of genes [Alizadeh et al., 2000;Eisen et al., 1998;Wen et al., 1998]. A disadvantage, however is that because prior biological knowledge is not incorporated, all measurements within the expression profile contribute equally to the analysis. This means that measurements that have little or nothing to do with distinguishing the groups of interest can confound the placement

of a sample into the correct category.

Hierarchical Clustering

Average-linkage hierarchical clustering was the first method to be used for ordering microarray data [Eisen et al., 1998]. This approach finds the pair of genes with the most similar expression profiles, and iteratively builds a hierarchy by pairing genes (or existing clusters) that are most similar. The resulting hierarchy is shown using dendrograms. Genes which have similar functions are often co-expressed and so cluster together. In this way, the functions of yet uncharacterised genes can be assigned based on the functions of genes with which they cluster. This can be extended to samples from similar origins, which will cluster together due to their expressing similar sets of genes [Perou et al., 1999].

K-means Clustering

K-means clustering requires a parameter, k, which represents the expected number of clusters [Tavazoie et al., 1999]. First, cluster centres are chosen at random. Using the distance metric, each profile is assigned iteratively to the cluster whose centre it is nearest to; then the cluster centre is recalculated depending on the profiles within the cluster.

Self-Organising Maps (SOMs)

Self-organising maps are topological neural networks [Tamayo et al., 1999]. Clusters are already organised into a "map" where clusters which are similar are topologically close together. The number and topology of the clusters are already specified. The clustering is similar to the k-means method except that the cluster centres are recalculated at each iteration based on the profiles within each cluster as well as the profiles in separate clusters.

1.6.3.2.2 Supervised Clustering

Supervised clustering techniques such as support vector machine (SVM) incorporate previous biological knowledge. For example, a supervised approach might be used to predict whether a gene is involved in ovarian carcinogenesis by comparing its expression profile to the profiles of both genes known to be involved and genes known not to be involved in ovarian carcinogenesis. Recent reports have demonstrated the ability of supervised classification to assign functions to genes [Brown et al., 2000] and to type leukaemia (myeloid vs. lymphoid) [Golub et al., 1999], based on microarray data.

The main disadvantage of supervised methods is that they are hypothesis driven. If a hypothesis is based on prior knowledge, supervised methods will help to accept or reject it. They cannot reveal the unexpected and do not form new hypotheses or data classifications. For example, if tumours fall into two unanticipated classes on the basis of their expression profiles, a supervised method will not be able to discover this. Another disadvantage is the possibility of misclassification of samples; supervised methods will not discover, in general, samples that were mistakenly labelled and used in, say, the training set.

1.6.3.3 Verification of Results

As microarray technology is still in its infancy, the techniques are continually being improved. Problems may arise such as probes not detecting the correct transcript species as a result of cross-hybridisation or adverse secondary structure. Verification of a subset of results by well-established techniques such as quantitative reverse transcription polymerase chain reaction (qRT-PCR) or northern hybridisation is important. QRT-PCR or Northern blotting is still considered standard to verify array data especially where only a few replicates are used. Comparative array analysis with other datasets available in the public domain is also helpful to verify array results.

1.6.4 The Application of Microarrays to Cancer Pathways

Gene expression profiling of cancers has expanded greatly in the past few years, and is becoming the most comprehensive approach for characterising human cancers at a molecular level. Gene expression profiling provides a global overview of the mechanisms and pathways involving numerous genes operating at a cellular level in many cancers, such as breast, colon, lung, stomach, ovary and head and neck. A vast amount of data has been generated, with some of these genes already known to be involved in the carcinogenic process, and many whose functions are still presently unknown. Similarly, the patterns of expression of genes with a known function can reveal information on novel phenotypic features of cells and tissues being studied.

1.6.4.1 Cancer Classification

Accurate classification of cancers is vital since correct treatment hinges on detailed knowledge of the primary tumour and presence or absence of metastatic spread. Until now, diagnosis has been determined histologically by appearance alone. The problem with this approach is that tumours, including tumour subtypes with similar histopathological appearance can have vastly divergent clinical outcomes and responses to treatment. Classification of tumours using gene expression profiles is providing a new dimension.

Golub and colleagues were the first to report that the expression patterns of diverse classes of genes contained information that could be used to classify tumours, specifically leukaemias [Golub et al., 1999]. They suggested a class discovery and a class prediction model. Class discovery defines previously unrecognised tumour subtypes, and class prediction refers to the assignment of tumours into defined categories according to their current state or future outcomes. 38 leukaemia cases were taken and analysed by self-organising maps (SOMs) using high-density oligonucleotides microarray containing almost 7,000 genes. A distinct molecular difference was shown between acute myeloid leukaemia (AML) and acute lymphoblastic leukaemia (ALL) based on the gene expression profiles. For class

prediction, tumours were assigned one of the two groups based on the class discovery. For example, one tumour had initially been diagnosed histopathologically as AML, but showed atypical morphology. Gene expression profiling demonstrated high expression of genes suggesting this was a tumour of mesenchymal origin. Subsequent cytogenetic re-analysis confirmed this to be a rhabdomyosarcoma, and treatment was changed accordingly. The authors stress that this method is an aid rather than a replacement to classic histopathological diagnoses.

A study by Alizadeh and colleagues [Alizadeh et al., 2000] investigated diffuse large B-cell lymphoma (DLBCL), an aggressive malignancy of mature B lymphocytes and the most common subtype of non-Hodgkin's lymphoma. Classification of this disease based on morphology has been largely unsuccessful, due to lack of agreement on histological appearance of possible subtypes. The clinical course is variable: although 40% of patients respond well to therapy, 60% do not, and ultimately succumb to their disease. Hierarchical clustering identified two molecularly distinct groups of DLBCL: the gene expression pattern of one group was indicative of B-cell differentiation pattern in germinal centres of lymph nodes, and the other group expressed genes induced during in vitro activation of peripheral B cells. Patients with the germinal centre B-like DLBCL had a significantly better overall survival rate. This study demonstrates that tumours can be classified into previously unrecognised but clinically significant subgroups.

Breast cancers are a heterogeneous group of tumours, with several different subtypes, with correspondingly variable prognoses. Perou et al [Perou et al., 1999] defined a further subtype of oestrogen-receptor negative group with basaloid features. Although histopathologists recognise the basaloid breast cancer subset, it has never been treated as a distinct clinical entity in clinical practice. This study, along with others [Jones et al., 2000;Jones et al., 2001;Tsuda et al., 2000] demonstrate that this group of tumours has a different gene expression profile and a metastatic pattern similar to ductal carcinomas in general. Microarrays can define the gene expression profile of this subset of ductal tumours, and other groups will most likely be discovered in the process.

Ross et al. [Ross et al., 2000] used cDNA microarrays to determine the expression of 8,000 genes within the 60 cell lines used in the National Cancer Institute screen for anticancer drugs. Hierarchical clustering analysis grouped cells of the same tissue origin together. Cell lines derived from leukaemia, melanoma, the central nervous system, colon, kidney and ovarian tissue clustered in distinct groups. However, breast cancer cell lines clustered in many different branches, suggesting a heterogeneous pattern of gene expression. Specifically, the profile obtained for two related breast carcinoma cell lines, MDA MB435 and MDA-N, which were derived independently from the same patient, was similar to that from melanoma cell lines. This suggested either that some breast cancers may have expression profiles exhibiting neuroendocrine features or that the cell lines are actually derived from a melanoma.

Cancers from divergent tissue types can also be classified into groups according to their gene expression profile. Taking gene expression profiles of 90 normal tissue samples, and 218 tumour samples containing 14 different tumour types, Ramaswamy et al showed that these cancers can be classified with an accuracy approaching 78% [Ramaswamy et al., 2001]. This group also showed that well differentiated tumours differ from poorly differentiated cancers, and very highly related tumour types are harder to classify. Another similar study looking at GEM profiles of 175 carcinomas from different tumour types used supervised machine learning algorithms to first filter genes not involved in tumour classification, then ranked the remaining genes according to their contribution to predict tumour types to classify these tumours [Su et al., 2001]. This approach allowed the prediction of tumour origin of 90% of the tumours studied.

1.6.4.2 Identification of Metastatic Markers

The pathways involved in invasion and progression of disease are poorly understood. Identification of genes responsible for metastasis can lead to the development of drugs aimed at preventing the expression of these genes and thus individualising patient treatment. The following microarray studies have shed some light on the identification of potential key regulators of human cancer.

A gene involved in the metastasis of melanoma, RhoC has been identified [Clark et al., 2000]. Highly metastatic melanoma cell lines were compared with non-metastatic lines. GEM profiling revealed a cluster of genes, including RhoC which were over-expressed in metastasis. Functional studies demonstrated that RhoC over-expression facilitated metastasis whereas dominant-negative RhoC inhibited metastasis in animal models. The authors concluded that RhoC may regulate invasion by triggering cytoskeletal organisation in response to signals stimulating cell motility. Drugs targeted towards RhoC could be used to reduce the spread of primary melanoma.

Prostate cancer is one of the most common cancers in men. It is histopathologically heterogeneous with a clinically diverse outcome, and is almost incurable once metastasised. Dhanasekharan et al have described a molecular signature for prostate cancer progression by comparing the gene expression profiles of normal prostate, primary prostate cancers and metastatic prostate samples [Dhanasekaran et al., 2001]. 55 genes were found to be over-expressed in metastatic specimens, of which the Polycomb protein enhancer of zeste homologue 2 (EZH2) was the most significantly up-regulated. In a follow-up study by the same group of researchers, EZH2 was further investigated and confirmed to be more highly expressed in metastatic prostate tissue than benign tissue [Varambally et al., 2002]. When expression of EZH2 was inhibited using small interfering RNA (siRNA), cellular growth of prostate cancer cells was significantly reduced. High EZH2 expression was correlated with poor prognosis. This suggests that EZH2 is a good marker for prostate cancer invasion and metastasis by its role in regulating cell growth. Drug treatment targeted against EZH2 may prevent metastatic spread of prostate cancer.

Breast cancer is one of the commonest cancers in women. The presence of lymph node metastases at the time of primary surgery is currently used to determine whether patients require adjuvant treatment in the form of cytotoxic or radiation therapy. These are associated with significant side-effects, and identifying in situ disease which is likely to metastasise is invaluable in improving quality of life for patients. Van't Veer and colleagues performed microarray studies on 78 breast cancers from patients with no lymph node metastases who had not received adjuvant

therapy ['t Veer et al., 2002]. These patients were followed up and a gene expression profile was generated to differentiate between tumours which went on to metastasise and those which did not. A "poor prognosis signature" comprising 70 differentially expressed genes was generated. This prognosis signature was then tested on another group of newly diagnosed breast cancer patients and accurately predicted disease outcome. The authors concluded that the signature for poor prognosis is already present in primary tumours, and this can be used at the time of initial surgery to predict survival. Such studies in the future may help to identify women with early breast cancer who will benefit from adjuvant chemotherapy after initial local treatment. However, there is currently a debate regarding the validity of the analysis used by V'ant Veer and colleagues to identify this high risk group.

Cancers in children can be devastating. Medulloblastoma is one such tumour; it is a highly metastatic cancer of the brain which is often difficult to distinguish histopathologically from other tumours. It is a lethal cancer due to its propensity for spread, and there is a need for reliable molecular markers that can distinguish this cancer from the non-metastatic types. MacDonald and colleagues used gene expression profiling to identify genes differentially expressed between the metastatic and non-metastatic medulloblastomas [MacDonald et al., 2001]. They found that platelet-derived growth factor receptor α (PDGFRα) and members of the Ras/MAPK (mitogen activated protein kinase) downstream pathway were slightly over-expressed in the metastatic tumours both at the RNA and protein levels. Functional studies demonstrated reduction of cell adhesion and migration in a medulloblastoma cell line in response to inhibitors of PDGFRα and the Ras/MAPK pathway. These findings suggest that PDGFRα and the Ras/MAPK pathway are involved in metastatic spread and that inhibitors of this pathway can be exploited for the treatment of medulloblastoma.

Potentially more effective drugs can be designed by using large-scale studies correlating gene expression profiles with drug responses [Scherf et al., 2000;Zembutsu et al., 2002]. Gene expression profiling can be used to monitor, validate and improve cancer drugs. Studies have been performed aimed at monitoring the metabolism and toxicology of anticancer therapies [Zhou et al.,

2002;Gerhold et al., 2001]. Other studies have identified the cellular targets of drugs and delineated the molecular mechanisms of drug action [Dimitroulakos et al., 2002;Chang et al., 2002]. These data should contribute to new drug design which is individually tailored to patients with minimum of adverse drug events.

1.6.4.3 Gene expression profiling of ovarian tumours

A series of studies have been performed investigating the gene expression profiles in ovarian cancers. These have been for the purpose of gaining insight into the progression pathway of ovarian carcinogenesis [Tapper et al., 2001;Matei et al., 2002;Jazaeri et al., 2003;Shridhar et al., 2001] and also for discovering gene products that can act as ovarian cancer specific markers and used for screening.

1.6.5 The Use of Microarrays in Ovarian Cancer

1.6.5.1 Understanding Ovarian Carcinogenesis

The molecular pathways crucial for initiation and progression of EOC remain largely unknown. Prognosis for EOC differs according to histological subtype, grade and stage of disease. Gene expression profiling may contribute to our knowledge and so improve patient outcome after treatment. Tapper and colleagues have attempted to define genes involved in ovarian cancer progression [Tapper et al., 2001]. They compared gene expression profiles from benign, local highly differentiated and advanced ovarian serous adenocarcinomas. The most significantly up-regulated gene between local, well-differentiated and benign tumours was cell division cycle 42 (cdc42) homologue, and that between local and advanced tumours was collagen type III alpha 1 (COL3A1) also known as type III or fetal collagen. Whilst this is a very good attempt at defining genes along the progression pathway, it assumes that benign tumours are a pre-malignant lesion, which is not the generally accepted view.

Ovarian cancer stage is one of the most important independent prognostic indicators; Shridhar et al compared GEM profiles of five stage I, two stage II and seven stage III ovarian cancers with normal ovary [Shridhar et al., 2001]. Hierarchical clustering

showed a clear distinction between normal and malignant tissues, although the tumour stages did not cluster together, and the gene expression profiles of early and late stage tumours were essentially the same, suggesting that a malignant signature is present at an early point in carcinogenesis. Comparative genomic hybridisation (CGH) analysis was performed in order to investigate whether any of the results from the GEM analysis could be correlated with genomic alterations, and showed that the main difference was regional gain and/or amplification in late-stage tumours. The inconsistency in the microarray and CGH data could be attributed to epigenetic mechanisms such as CpG island methylation, which have previously been reported to be early events in ovarian tumorigenesis [Cheng et al., 1997]. These are early studies with small sample sizes and need to be confirmed in larger prospective studies where known prognostic indicators e.g. tumour grade are taken into account. However, these studies do suggest that genes identified by microarrays play an important role in the future in the prognostic classification of early ovarian cancer.

Well differentiated tumours are generally thought to have a better prognosis than undifferentiated cancers. Two groups [Matei et al., 2002;Jazaeri et al., 2003] compared the GEM profiles of grade 1 and grade 3 serous adenocarcinomas. Different genes were identified by each group, with serine/threonine protein kinase 15 (STK15) and runt-related transcription factor 2 (runx2 or OSF2) being the two most up-regulated genes in grade 3 compared to grade 1 tumours.

1.6.5.2 Ovarian Cancer Biomarkers

Ovarian cancer presents late due to a lack of symptoms at an early stage, and the lack of an effective screening strategy. It is likely that advanced ovarian cancer is a different disease from limited disease. The initial genetic events leading to uncontrolled cell growth in tumours that present as advanced disease might also confer to those cells the propensity for early invasion and aggressive clinical behaviour. Therefore, screening strategies to detect early disease may not be that successful. Having said that, CA125 is currently the only marker in general use for ovarian cancer screening, but is neither sensitive nor specific enough to be used as a population based screening tool.

The circulation tumour antigen, CA125 is currently the only marker in general use for ovarian cancer screening, but is neither sensitive nor specific enough to be used as a population based screening tool. CA125 is elevated in only 50% of stage I tumours, so many microarray studies have been directed at discovering new potential biomarkers for ovarian cancer which can be used either alone or in conjunction with CA125 or other molecular markers.

Welsh et al compared normal ovarian tissue with serous papillary adenocarcinomas using oligonucleotide arrays and also included ovarian cancer cell lines, and RNA from endothelial and activated B cells to control for blood vessels and infiltrating immune cells [Welsh et al., 2001]. Genes most highly expressed in ovarian tumours were CD24, HE4, CD9 with tumour-associated antigen GA773-2 (TACSTD1), cytokeratins 7,8,18 and 19 and mucin-1 (MUC-1) also being highly up-regulated. Ismail et al also compared normal ovary with papillary serous ovarian adenocarcinomas, but in this case using primary cultures [Ismail et al., 2000]. There is always some concern where cell lines are used as short-term culture may favour the growth of only a subset of epithelial cells. However, they identified osteoblast specific factor-2 (OSF-2) as a highly ranked gene in primary ovarian cancers. Follow-on studies are needed to verify that these genes are up-regulated only in primary cancers and not, as is the case for CA125, in a multitude of benign conditions.

Three other studies have also compared normal ovarian tissue with ovarian cancers. HE4, matrix metalloproteinase 7 (MMP7 or PUMP1), progesterone binding protein, ryudocan, MUC-1, membrane protein E16 and BA46 were reported as over-expressed in the cancer group. However, the cancers represented a mixed population of different EOC subtypes and stages, each of which have widely varying clinical outcomes. Therefore only broad conclusions can be drawn from these studies [Wang et al., 1999;Schummer et al., 1999;Tonin et al., 2001].

The only study so far to verify biomarkers identified in microarray experiments serologically investigated the potential use of prostasin [Mok et al., 2001b]. This potential marker was found to be raised in the sera of patients with serous ovarian cancer and only marginally raised in mucinous or LMP tumours. It proved to have low

sensitivity in early-stage disease, and was found to have no correlation with CA125. The authors suggest combining the two markers could increase the sensitivity; based on screening of 37 patients and 100 controls, a sensitivity of 92% was achieved (95% confidence interval (CI) 78.1-98.3%) and a specificity of 94% (95% CI 87.4-97.7%). This compared with sensitivities of 51.4% (95% CI 34.4-68.1%) for prostasin and 64.9% (95% CI 47.5-79.8%) for CA125 for a specificity of 94% for prostasin. The authors only tested this marker in patients with benign or malignant gynaecological conditions. Prostasin can be found in eight normal human tissues: prostate, kidney, liver, pancreas, salivary gland, lung, bronchus and colon, so whether it functions as a good screening marker remains to be determined.

Microarrays contain thousands of genes and ESTs which represent many different gene functions and are involved in numerous pathways. Not all of these are relevant to the study of ovarian cancer. Based on findings from SAGE analysis of up-regulated genes in ovarian cancer [Hough et al., 2001] and other cDNA array data, Sawiris et al have produced the "Ovachip" which consists of 516 cDNA clones [Sawiris et al., 2002]. This specialised array does not include irrelevant genes which may contribute to biological noise affecting data analyses. However, this assumes that all genes thought to be involved in ovarian carcinogenesis have been identified, and may miss vital new genes as yet not discovered.

Many studies have investigated the gene expression profile of ovarian cancer using microarray technology. Most studies use snap frozen fresh tissue for analysis, commonly ovarian serous adenocarcinomas. Results from cell lines give varying results, especially as there are significant differences in the gene expression profiles of cell lines according to the length of time they have been in culture [Santin et al., 2004].

A wealth of data has been generated from studies investigating the gene expression profiles to differentiate serous adenocarcinoma from normal ovarian tissue, with many upregulated genes being involved in cell growth, differentiation, adhesion, apoptosis and migration [Donninger et al., 2004;Meinhold-Heerlein et al., 2005;De et al., 2004]. Specifically, creatine kinase B [Huddleston et al., 2005], KLK10

[Shvartsman et al., 2003], osteopontin [Kim et al., 2002b] and FGF2 [De et al., 2004] have been proposed as putative novel targets to be further investigated as useful markers for ovarian cancer.

Patients with stage III/IV ovarian cancer who respond well to chemotherapy have an improved survival than those who respond badly. Studies have identified a gene expression signature which distinguishes short-term (<2-3 years) from long-term (>7 years) survivors [Berchuck et al., 2005], with many of the genes identified being involved with immune function [Lancaster et al., 2004]. The expression pattern for long-term survivors was similar to a set of stage I/II cancers, suggesting a common favourable biology. In addition The MAL gene, known to be associated with chemoresistance was highly expressed in short-term survivors[Berchuck et al., 2005].

One of the aims of identifying putative markers is to test them either individually or as a on a panel of serum markers in order to test whether they can be used to for screening of early stage disease. Mor et al tested osteopontin, insulin-like growth factor-II, leptin and prolactin and found that any protein individually could not distinguish patients with ovarian cancer from those unaffected, but in combination could detect the disease with a sensitivity of 95%, a specificity of 95% and a positive predictive value of 95% [Mor et al., 2005].

1.6.6 Access to Array Databases

With the enormous amount of data generated from DNA microarray experiments, it would be reasonable to conclude that this information should be available in the public domain, in order that a re-analysis may be performed by other researchers, to verify the original claims, but more importantly to compare these findings with their own datasets. This would be best accomplished by posting raw data in a centralised public database. In fact, some DNA databases have been generated, due to the requirement of certain scientific journals that DNA sequence data are sent to GenBank prior to submission of an article.

Obviously, not all data generated can be compared equally because different arrays may be used in different experiments, with different probes for the same gene.

Accession numbers are used to identify genes, and these vary between organisations according to which array is being used. Therefore it is imperative to confirm that the same genes are being compared. Normalisation of arrays may differ, and this must be checked by a trained bioinformaticist. However, even if global normalisation is implemented, the actual intensity values for the same gene may vary.

Difficulties arise when comparing high-density oligonucleotide arrays and cDNA arrays. Oligonucleotide arrays generate robust data and more reproducible results than cDNA arrays, where the absolute hybridisation intensities often vary due to differences in the amount of DNA deposited on the array for various genes.

1.7 Screening for Ovarian Cancer

Given the poor prognosis associated with the development of advanced ovarian cancer and the clear increased survival at early stage disease, there has been much enthusiasm for the development of effective screening strategies aimed at early detection. There is currently no evidence to justify mass population screening; however, concentrating on subpopulation screening, identification of novel serological biomarkers, and multimodality screening with transvaginal ultrasound (TVS) and novel imaging, it is hoped that these refinements may eventually translate into a reduction in ovarian cancer mortality.

Many features of ovarian cancer have led to the frustrations encountered in attempting to screen for the disease; the anatomical location of the ovaries means they cannot be directly inspected, and epithelial ovarian cancers have no defined precursor lesion and a poorly defined natural history. The time required for localised disease to progress to disseminated disease is not known, so it is unclear what the screening interval should be. The low prevalence of ovarian cancer in the general population means that screening must have a very high specificity in order to avoid unnecessary surgical interventions. Suboptimal specificity will lead to high financial and personal costs that may not be acceptable when balanced against the benefits of screening. Therefore routine screening for ovarian cancer is currently not recommended, but studies are underway to identify new screening modalities and novel applications for screening regimens in high-risk populations.

Serum CA125 is the tumour marker which has been most extensively studied in ovarian cancer screening. Bast et al discovered that serum levels of CA-125 were elevated in 82% of women with advanced ovarian cancer but in less than 1% of presumed healthy women and it was suggested that it could be used as a serum marker for EOC [Bast, Jr. et al., 1983]. Soon afterwards, the JANUS study showed encouraging evidence that CA125 levels increase for a period of time before the development of clinically apparent ovarian cancer [Zurawski, Jr. et al., 1988]. Despite these findings, the usefulness of CA125 in detecting preclinical ovarian cancer is limited by a lack of sensitivity in stage I disease and poor specificity in that the

marker is elevated in numerous benign and malignant conditions such as endometriosis, fibroids and any condition leading to peritoneal inflammation [Jacobs and Bast, Jr., 1989].

Despite these drawbacks, two large European screening trials have been completed that evaluate CA125 as a first-line test, with TVS being performed in cases where CA125 is raised. The first of these trials was reported by Einhorn [Einhorn et al., 1992]. In this trial, 5500 women were screened initially with CA125; 175 women had elevated levels and were followed up with serial CA-125 and TVS. 16 laparotomies were performed on those with abnormal results, with 6 cases of ovarian cancer detected, distributed evenly between stages I, II, and III. Three cases of ovarian cancer were diagnosed in the control group. The other large study was reported by Jacobs [Jacobs et al., 1993] which involved 22,000 healthy postmenopausal women. Again patients with elevated CA125 underwent subsequent TVS. 11 ovarian cancers were diagnosed for 41 laparotomies performed. However, eight women with negative screens subsequently developed ovarian cancer. As CA125 has insufficient specificity, sensitivity, and predictive values, algorithms incorporating age and rate of change of CA125 have been developed in order to improve the performance of CA125 as a screening tool [Skates et al., 1995].

TVS has been investigated as a tool for ovarian cancer screening. Van Nagell et al. [van, Jr. et al., 1990] reported on one thousand asymptomatic women over forty years of age who underwent TVS screening. Of these, 31 had abnormal scans and 24 patients underwent laparotomy. One Krukenberg tumour was found, but no cases of primary ovarian carcinoma were detected. Given the unacceptable number of laparotomies performed to detect one case of malignancy, subsequent studies have focused on postmenopausal populations and defining ovarian scan morphology [van, Jr. et al., 1991;DePriest et al., 1993]. The same researchers have reported on the results of annual TVS screening performed on 14,469 asymptomatic women [van, Jr. et al., 2000]. In this report, 57,214 scans were performed, and 180 patients underwent laparotomy for 17 ovarian cancers detected: 11 stage I, 3 stage II, and 3 stage III. In this study, TVS as a screening modality gave a sensitivity of 81%, specificity of 98.9%, positive predictive value of 9.4%, and negative predictive value

of 99.97%. Excluding cases of nonepithelial and low malignant potential (LMP) tumours, the survival of patients with ovarian cancer in the annually screened population was 92.9% at 2 years and 83.6% at 5 years. This study was, however, not randomised so any assessment regarding the possible reduction in risk of mortality cannot be made. However, the financial and personal cost of annual TVS screening and subjecting 10 women to a surgical intervention to identify one cancer, make population screening with TVS as a single modality unacceptable.

More recent screening trials have focused on multimodal screening designs. Jacobs reported the results of the first completed randomised trial of ovarian cancer screening [Jacobs et al., 1999]. This prospective study included 22,000 postmenopausal women aged over 45 who were randomised either to screening (10,997 women) or follow-up with no screening (10,958 women). The primary screen in this study consisted of serum CA125 measurement. Women who were found to have a CA125 level of over 30U/ml were asked to attend for an ultrasound scan. At the beginning of the study the scans were performed abdominally and when TVS became available this was the preferred route. 468 women with a CA125 level above 30U/ml underwent a total of 781 scans. 29 women underwent surgery, with 6 cases of ovarian cancer diagnosed, 3 stage I/II, and 3 stage III cancers. The remaining 23 women had false-positive results with benign conditions such as benign ovarian tumours and fibroids. Four operations were performed for each cancer detected, giving a positive predictive value of 21. A further 10 women developed ovarian cancer after the period of screening during the 8 year follow-up, bringing the total to 16 in the screened group. 20 women in the control arm were diagnosed with ovarian cancer. The prevalence in the 2 groups was not significantly different, with stage distribution and histological type of cancer being similar, but the cancers in the screened group were of a lower grade. Although the mortality did not differ significantly between the 3 groups, this study was conducted primarily as a basis of the feasibility of multi modal screening with CA125 and ultrasound scanning. Compliance was good, with 86% of women attending for at least one screen. This has prompted the initiation of a large randomised trial, the UK Collaborative Trial of ovarian cancer screening (UKCTOCS). The aim is to recruit 200,000 postmenopausal women aged between 50 and 74 years who will be randomised in a

1:1:2 ratio to one of 3 arms: ultrasound scan alone, multimodal screening using an algorithm for risk of ovarian cancer and a control group. The screened groups will undergo 6 screens at annual intervals. The 2 screening strategies will be compared in order to evaluate the benefit of adding CA125 measurement to the screening procedure. The primary end point will be ovarian cancer mortality 7 years after randomisation, with the data being accrued by postal questionnaire and through the cancer registry. The results of this trial will provide invaluable information as to the benefit of screening employing current tools, in the general population.

Serum markers other than CA125 have been evaluated. Lysophosphatidic acid (LPA) was assessed in a small pilot series; LPA levels were elevated in 90% of stage I ovarian cancers and in 100% of patients with stages II–IV [Xu et al., 1998]. Larger studies are needed to fully evaluate the usefulness of LPA.

To date, strategies for ovarian cancer screening have had inadequate sensitivity and specificity to justify population screening. Efforts aimed at the development of new screening strategies, identification of novel serum biomarkers and preventative measures are urgently needed; if successful, these strategies and measures could have a substantial impact on the lives of women.

AIMS OF THIS THESIS

Epithelial ovarian cancer has the highest mortality rate of the gynaecological cancers. Approximately 75% of women have advanced stage disease at the time of diagnosis. Despite aggressive surgery and improvements in chemotherapeutic regimes, the prognosis for these women has remained poor over the past 20 years, at around 25% 5 year survival. This is due in part to the lack of effective prevention or molecular markers for early stage detection. When detected at stage I, the survival rate is around 95%. Clearly, improvements in early detection of this disease are paramount.

The aim of this thesis is to identify a gene expression profile for serous epithelial ovarian cancer which contributes to the understanding of ovarian cancer and which reveals putative novel tumour markers.

CHAPTER 2

MATERIALS AND METHODS

2.1 Clinical Samples

2.1.1 Collection of Clinical Samples

Clinical samples were obtained at the time of surgery at both University College and Royal Free Hospitals. As soon as the specimen was removed from the patient, a small representative biopsy from the tissue concerned was cut, placed in a sterile container and then immediately into dry ice (at −80°C). The samples were labelled then stored in a −80°C freezer until the histology report was available. 47 samples of ovary and omentum in total were collected. All patients were undergoing primary staging laparotomies prior to chemotherapeutic intervention. This study was only concerned with investigating stage IIIC malignant serous cystadenocarcinomas where the corresponding omentum was available; therefore many samples had to be discarded as the pathology report showed they represented other histologies. The details are summarised in Table 2.1. Eleven patients were diagnosed with stage IIIC serous adenocarcinoma of the ovary and were therefore used in the study; 18 patients had other malignant histology and 10 patients had benign histologies. In addition, biopsies of 8 normal ovaries from patients undergoing elective surgery (total abdominal hysterectomy and bilateral salpingoophorectomy) for benign conditions were collected, where no abnormality was detected in the ovaries, uterus, cervix and fallopian tubes.

Ovarian epithelium was macrodissected from the underlying stroma in the normal ovaries for subsequent analysis. For the real-time quantitative RT-PCR data three serous tumours of low malignant potential were used in addition, which were obtained from the tumour bank at the Royal Free Hospital School of Medicine and these were gifts from Dr. Christopher Perrett.

Sample	Histology	Stage	Summary
1	Mucinous cystadenocarcinoma of the ovary	IIIC	M
2	Serous cystadenocarcinoma of the ovary	IIIA	✓
3	Clear cell carcinoma of the ovary	IIIC	M
4	Normal ovary	-	N
5	Benign serous adenoma of the ovary	-	B
6	Primary fallopian tube carcinoma	IIIC	M
7	Mucinous cystadenocarcinoma of the ovary	IIIC	M
8	Normal ovary	-	N
9	Normal ovary	-	N
10	Benign fibroadenoma of the ovary	-	B
11	Serous cystadenocarcinoma of the ovary	IIIC	✓
12	Serous cystadenocarcinoma of the ovary	IIIC	✓
13	Mucinous cystadenocarcinoma of the ovary	IIC	M
14	Borderline serous adenocarcinoma of the ovary	IIC	M
15	Serous cystadenocarcinoma of the ovary	IIIC	✓
16	Serous cystadenocarcinoma of the ovary	IIIC	✓
17	Normal ovary	-	N
18	Serous cystadenocarcinoma of the ovary	IIIC	✓
19	Primary fallopian tube carcinoma	IIIC	M
20	Normal ovary	-	N
21	Benign serous adenoma of the ovary	-	B
22	Benign mucinous adenoma of the ovary	-	B
23	Mucinous cystadenocarcinoma of the ovary	IIIC	M
24	Serous cystadenocarcinoma of the ovary	IIIC	✓
25	Endometrioid Adenocarcinoma of the ovary	IIIC	M
26	Endometrial sarcoma	II	M
27	Benign serous adenoma of the ovary	-	B
28	Serous cystadenocarcinoma of the ovary	IIIC	✓
29	Endometrioid Adenocarcinoma of the ovary	IIIC	M
30	Normal ovary	-	N
31	Borderline serous adenocarcinoma of the ovary	I	M
32	Mucinous cystadenocarcinoma of the ovary	IIIC	M
33	Mucinous cystadenocarcinoma of the ovary	IIIC	M
34	Serous cystadenocarcinoma of the ovary	IIIC	✓
35	Benign serous adenoma of the ovary	-	B
36	Mucinous cystadenocarcinoma of the ovary	IIIC	M
37	Benign mucinous adenoma of the ovary	-	B
38	Serous cystadenocarcinoma of the ovary	IIIC	✓
39	Normal ovary	-	N
40	Normal ovary	-	N
41	Brenner tumour of the ovary	-	B
42	Benign fibroadenoma of the ovary	-	B
43	Clear cell carcinoma of the ovary	IIIC	M
44	Serous cystadenocarcinoma of the ovary	IIIC	✓
45	Benign serous adenoma of the ovary	-	B
46	Borderline mucinous adenocarcinoma of the ovary	IA	M
47	Mucinous cystadenocarcinoma of the ovary	IIA	M

Table 2.1. Summary of all ovarian samples collected from theatre.

Histologies were available from the Pathologist approximately 1 week after collection from theatre. Those samples showing entirely normal ovaries with no co-existing pathology were included, as were stage IIIC serous cystadenocarcinomas of the ovary where samples of metastasis (omentum) were also available.

✓ Malignant histology suitable for this study (11)
B Benign histology (10)
M Other malignant histology (18)
N Normal ovary (8)

The number of suitable samples included 11 pairs of serous cystadenocarcinoma of the ovary and the corresponding omentum from the same individual (Table 2.2) and 8 normal ovaries (Table 2.3). These samples were used for analysis in this study. Ethical approval was granted by the University College Hospitals Trust, and preoperative informed consent was obtained from each patient.

In total, 73 samples of ovary and omentum were collected from 47 patients. 39 patients underwent primary debulking surgery for presumed ovarian cancer prior to receiving chemotherapy. I was able to obtain both ovary and omentum samples from 26 patients, the remaining 13 patients I obtained only ovarian biopsies. Of the 26 patients, 11 had serous adenocarcinoma and were used in the experiments. 8 patients underwent hysterectomies for benign conditions and were used as the normal ovarian controls.

Sample	Histology	Tissue adequate quality for analysis	Designation for purpose of study
2	Serous cystadenocarcinoma of the ovary		
11	Serous cystadenocarcinoma of the ovary	✓	O1, M1
12	Serous cystadenocarcinoma of the ovary		
15	Serous cystadenocarcinoma of the ovary	✓	O2, M2
16	Serous cystadenocarcinoma of the ovary	✓	O3, M3
18	Serous cystadenocarcinoma of the ovary		
24	Serous cystadenocarcinoma of the ovary	✓	O4, M4
28	Serous cystadenocarcinoma of the ovary		
34	Serous cystadenocarcinoma of the ovary	✓	O5, M5
38	Serous cystadenocarcinoma of the ovary		
44	Serous cystadenocarcinoma of the ovary	✓	O6, M6

Table 2.2. Samples (n=11) with histology showing stage IIIC serous cystadenocarcinoma of the ovary appropriate for analysis from initial group collected
All samples were grade 3, stage IIIC cancers. O=primary ovarian cancer, M=metastasis

Sample	Histology	Tissue adequate quality for analysis	Designation for purpose of study
4	Normal ovary	✓	N1
8	Normal ovary		
9	Normal ovary		
17	Normal ovary	✓	N2
20	Normal ovary	✓	N3
30	Normal ovary		
39	Normal ovary	✓	N4
40	Normal ovary	✓	N5

Table 2.3. Samples (n=8) with normal histology identified for analysis from initial group collected
N=normal ovary

2.1.2 Histopathological Verification

Each piece of tissue used for subsequent microarray analysis was embedded in 10% buffered formalin and 4µm thick paraffin sections were cut using a microtome in the laboratory of Dr. Ming Du. These sections were stained with haematoxylin and eosin (H&E) stain as below. H&E-stained sections were initially examined and verified histopathologically to be stage III serous adenocarcinomas of the ovary, then reviewed by the Pathologist Dr. Flanagan at the MDT meeting and the histology confirmed. The percentage of tumour cells in each tissue block was estimated; all samples comprised at least 70% tumour, except one omental sample which had 5% tumour content. The normal ovarian samples were verified to be free of any pathology, including benign cysts.

2.1.2.1 Haematoxylin and Eosin Staining Protocol

1. Sections were dewaxed and re-hydrated.
2. They were then placed in haematoxylin solution for 5-10 minutes.
3. Sections were washed in running H_2O.
4. Sections were differentiated in 1 % acid-alcohol and then washed well in H_2O.
5. Sections were rinsed ('blued') in ammonia H_2O for 1 minute.
6. Then briefly rinsed in distilled H_2O.
7. Counterstaining was performed with eosin for 2-5 minutes.
8. Sections were washed well in H_2O.
9. Finally, sections were dehydrated, cleared and mounted in neutral mounting medium.

2.1.3 Microdissection

Frozen tissue was taken and mounted in Tissue-Tek® O.C.T. compound embedding medium (Electron Microscopy Sciences, Washington, USA). Consecutive sections 6µm thick of frozen tissue were cut in a cryomicrotome at -20°C. The first and final sections were stained with H&E as described above and were used to identify the location of target cells. Microdissection of neoplastic cells was carried out using

sterile needles under a dissecting microscope. Target cells were recovered from a minimum of 10 consecutive sections. The microdissected cells were transferred into a sterile microtube containing RNAlater® which immediately inactivates RNases and stabilizes RNA within tissues or cells. RNA is stable for 1 day at 37°C, 1 week at 25°C, 1 month at 4°C or indefinitely at -20°C.The cells were then either used the same day for RNA extraction, or frozen at -20°C for later use.

2.2 RNA Sample Preparation

2.2.1 RNA Extraction

30mg of tissue was sharply dissected from the tissue mass, placed in 600µl RNeasy lysis buffer (buffer RLT, Qiagen, UK) and allowed to thaw on ice. It was then disrupted and homogenised using a rotor-stator homogeniser for up to 1 minute at 20 second bursts, and further homogenised by passing the lysate through a QIAshredder column by centrifugation (14,000g for 2 minutes). Total RNA was extracted from this tissue using the RNeasy Mini protocol (Qiagen, UK). The lysate was further centrifuged at 14,000g for 3 minutes, and the supernatant transferred into a new microcentrifuge tube and the pellet discarded. 600µl of 70% ethanol was added and the solution immediately mixed. 700µl of the sample at a time was applied to an RNeasy mini spin column placed in a 2ml collection tube and centrifuged at 14,000g for 15 seconds. After each centrifugation step the flow-through was discarded. The column was washed once with 700µl buffer RW1 and then twice with 500µl buffer RPE. The first 2 washes were centrifuged at 14,000g for 15 seconds, and the final wash for 2 minutes in order to dry the RNeasy silica-gel membrane. Total RNA was eluted by applying 50µl diethylene pyrocarbonate (DEPC)-treated distilled water (add 0.1% DEPC (v/v), shake, incubate at 37°C for 16 hours, then autoclave), and centrifuging at 14,000g for 1 minute.

2.2.2 RNA Quantification Using the Agilent 2100 Bioanalyzer

The concentration and purity/integrity of the RNA was checked using the Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA) and RNA 6000 LabChip® kit (Caliper Technologies Corp., CA, USA). The Bioanalyzer is shown in Figure 2.1. The traditional methods for analysing the quality of total RNA preparations is by agarose gel electrophoresis using ethidium bromide staining; the concentration of the purified RNA is calculated from the UV absorbance at 260nm using a UV spectrophotometer and the purity of RNA measured by the ratio of the absorbencies at 260nm and 280nm, taking an absorbance of $1cm^{-1}$ to be equivalent to 40µg/ml RNA. The main disadvantage of these techniques is that significant amounts of sample are required,

and RNA extraction of fresh tissue does not always yield much RNA. Therefore the Bioanalyzer was used which offers several advantages; a small amount of sample is required, there is minimal manual handling of the RNA sample, and the data is analysed quickly and accurately. The RNA 6000 LabChip kit was used in conjunction with the Bioanalyzer to measure both the quantity and the integrity and purity of the RNA samples. The ratio of the 18s and 28s ribosomal RNA bands is determined and displayed with the RNA quantitation data on the electropherogram. RNA was initially checked using both gel electrophoresis then the spectrophotometer, and the Agilent bioanalyzer in order to ensure replication of results. The bioanalyzer was used in all subsequent analyses.

Figure 2.1. Agilent 2100 Bioanalyzer machine and accompanying laptop.
Taken from www.agilent.com.

All chips were prepared according to the instructions in the chip preparation protocol provided with the RNA 6000 LabChip kit. Figure 2.2 shows the RNA 6000 LabChip. The kit contains most of the required components: 25 chips, syringe, spin filters, and reagents: sample buffer, gel matrix and dye concentrate. The reference ladder used was the RNA 6000 ladder (Ambion, Inc. Huntingdon, UK). Firstly, the gel-dye mix was prepared by mixing 65 µl of the gel matrix with 1 µl of dye concentrate, and centrifuged at 13000g for 10 minutes. 9.0 µl of the gel-dye was pipetted into the three gel-dye wells. 5 µl of the sample buffer (RNA 6000 Nano Marker) was added to each sample well and the ladder well. 1 µl of RNA sample (up to 12 samples can be run on the chip simultaneously) in the concentration range of 10 to 500 ng/µl were loaded into the sample wells of the chip. Finally, 1 µl of heat denatured RNA 6000 ladder was pipetted into the assigned ladder well. The chip was then vortexed and run on the Agilent 2100 bioanalyzer.

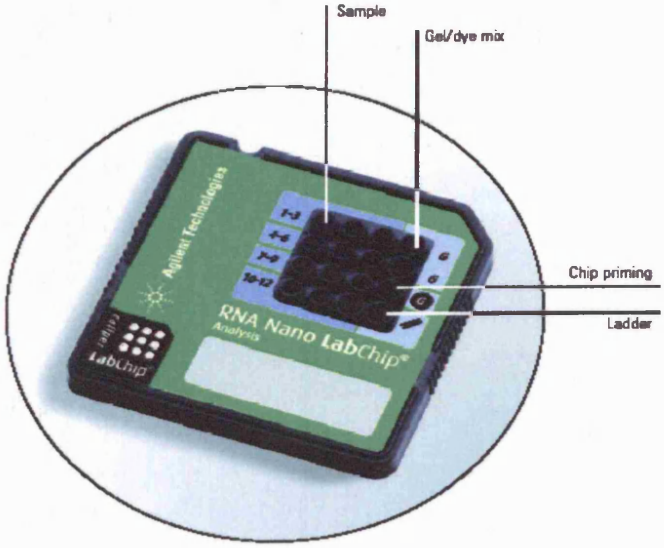


Figure 2.2. RNA 6000 LabChip
Taken from www.agilent.com.

The RNA 6000 ladder standard is run on every chip from the specified ladder well and is used as a reference for data analysis. The RNA 6000 ladder contains six RNA fragments ranging in size from 0.2 to 6 kb at a total concentration of 150 ng/µl. The software automatically compares the 12 unknown samples to the ladder fragments to determine the concentration of the unknown samples and to identify and calculate the ratio of the peak areas of the ribosomal bands, 18S/28S. An ideal total RNA preparation would result in a ratio of 2. Variability in this ratio may indicate partial degradation of the sample by ribonuclease contamination. Figures 2.3, 2.4, 2.5 and 2.6 show representative electropherograms for the RNA 6000 ladder, high quality RNA, degraded RNA and genomic DNA contamination, respectively.

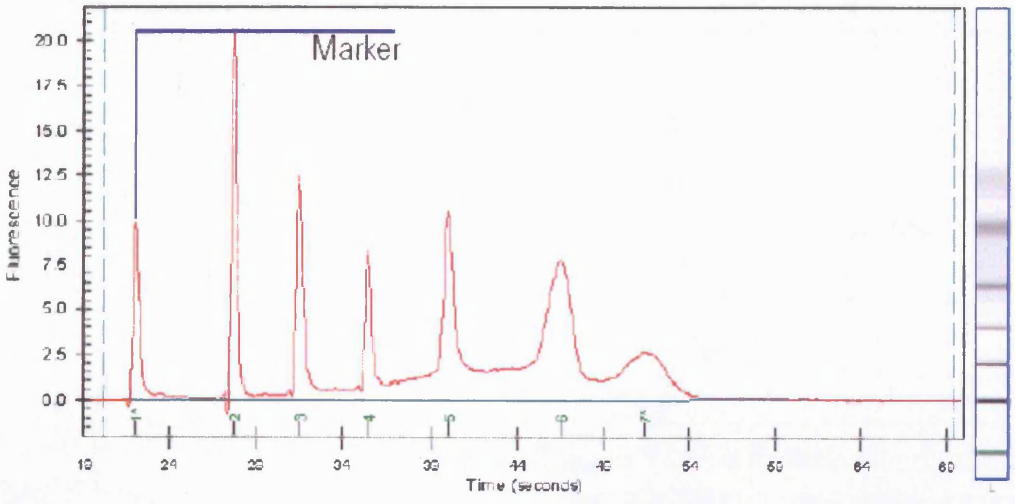


Figure 2.3. Electropherogram of RNA 6000 ladder, and gel-like image (right).

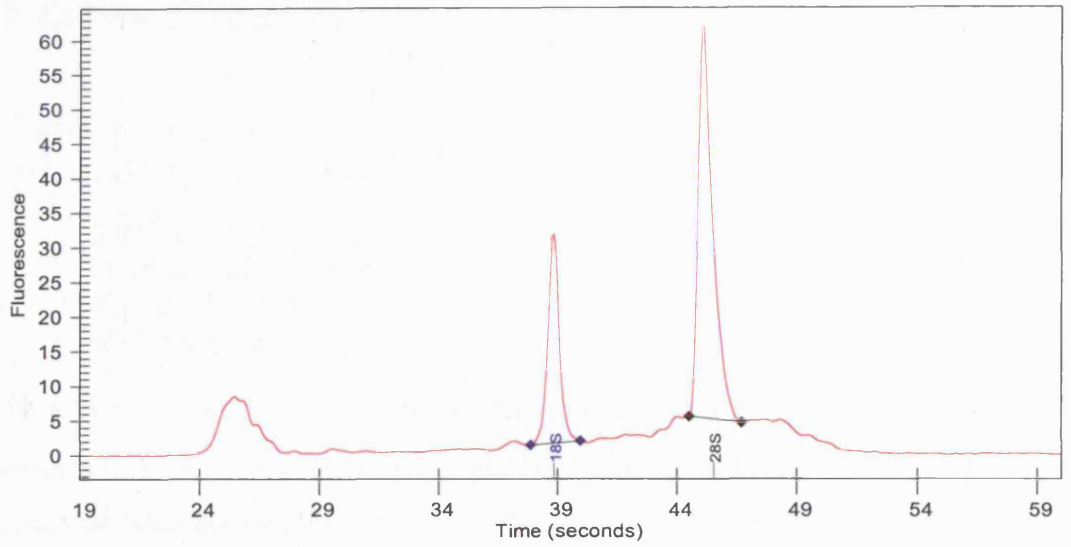


Figure 2.4. Electropherogram of high quality RNA

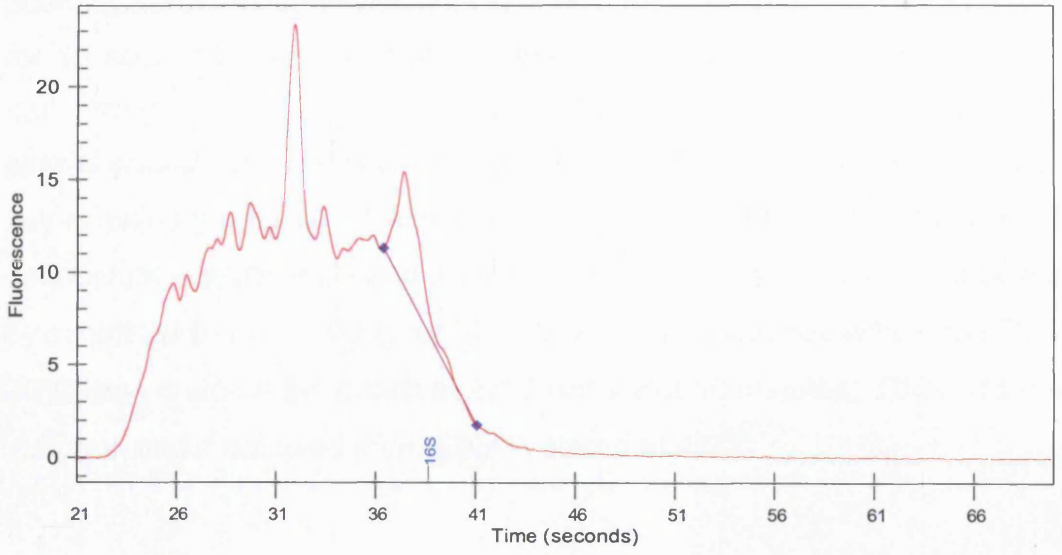


Figure 2.5. Electropherogram of degraded RNA

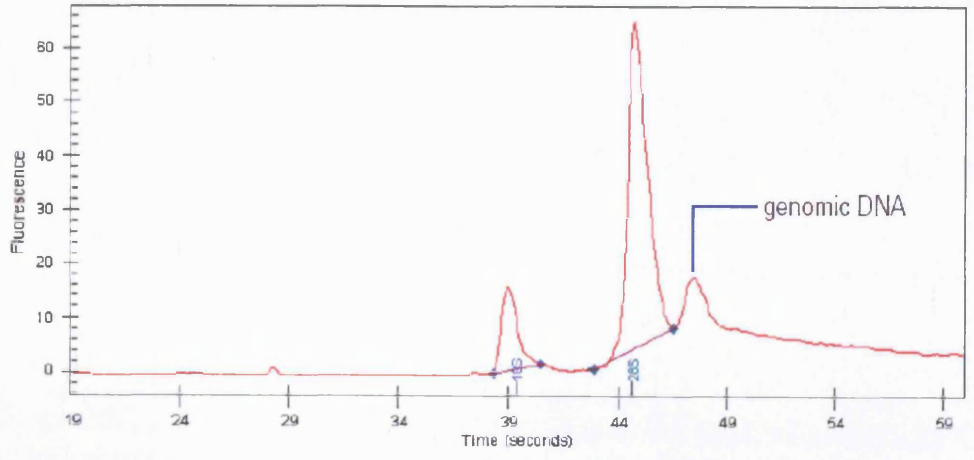


Figure 2.6. Electropherogram of genomic DNA contamination

2.2.3 DNase Treatment of RNA

Contaminating DNA was removed from RNA by treatment with DNase 1 (Promega, UK). The following protocol was used:

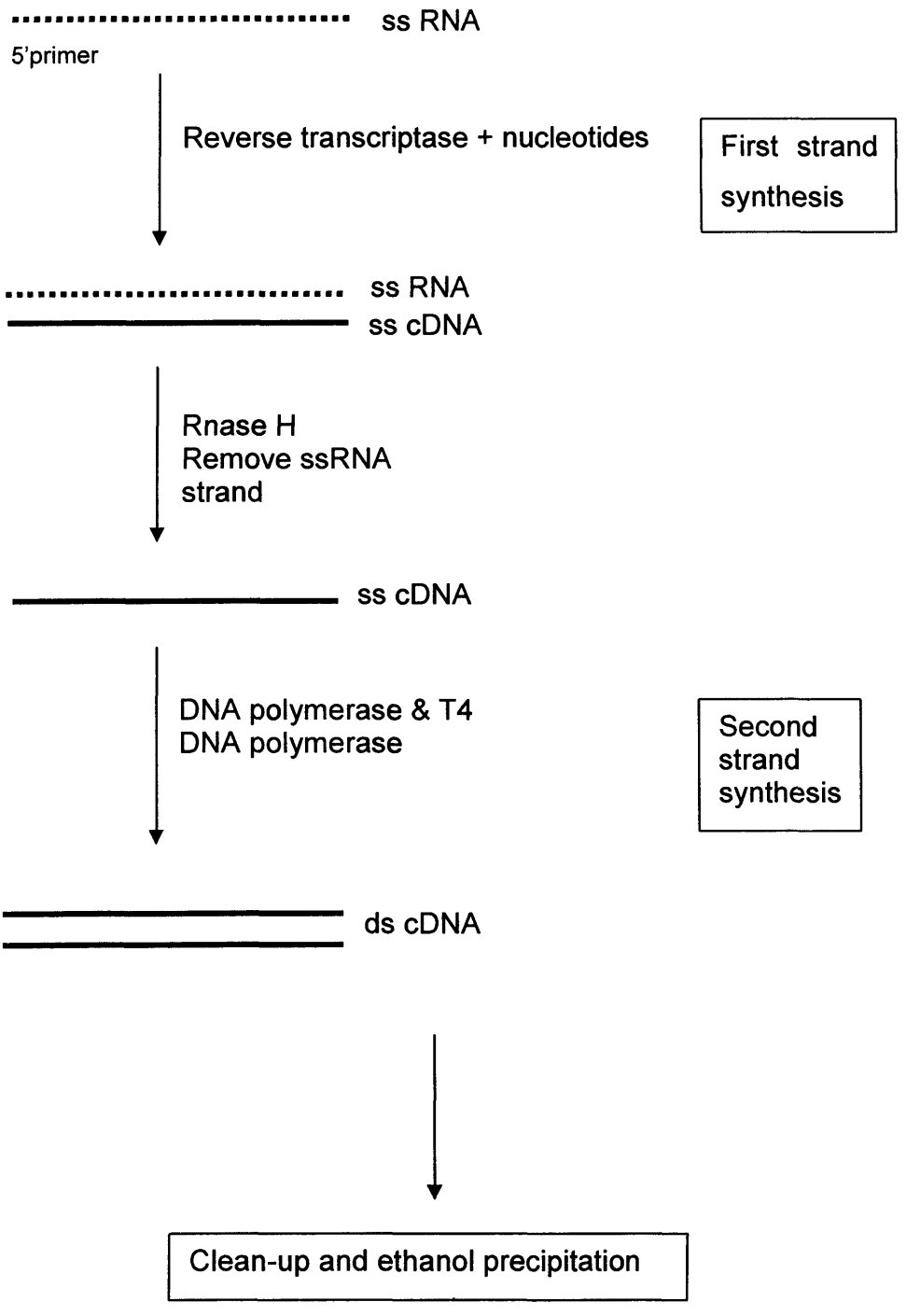
RNA (1µg/µl)	10µl
DEPC-treated water	7µl
DNase 1 (1U/µl)	1µl
DNase buffer	2µl

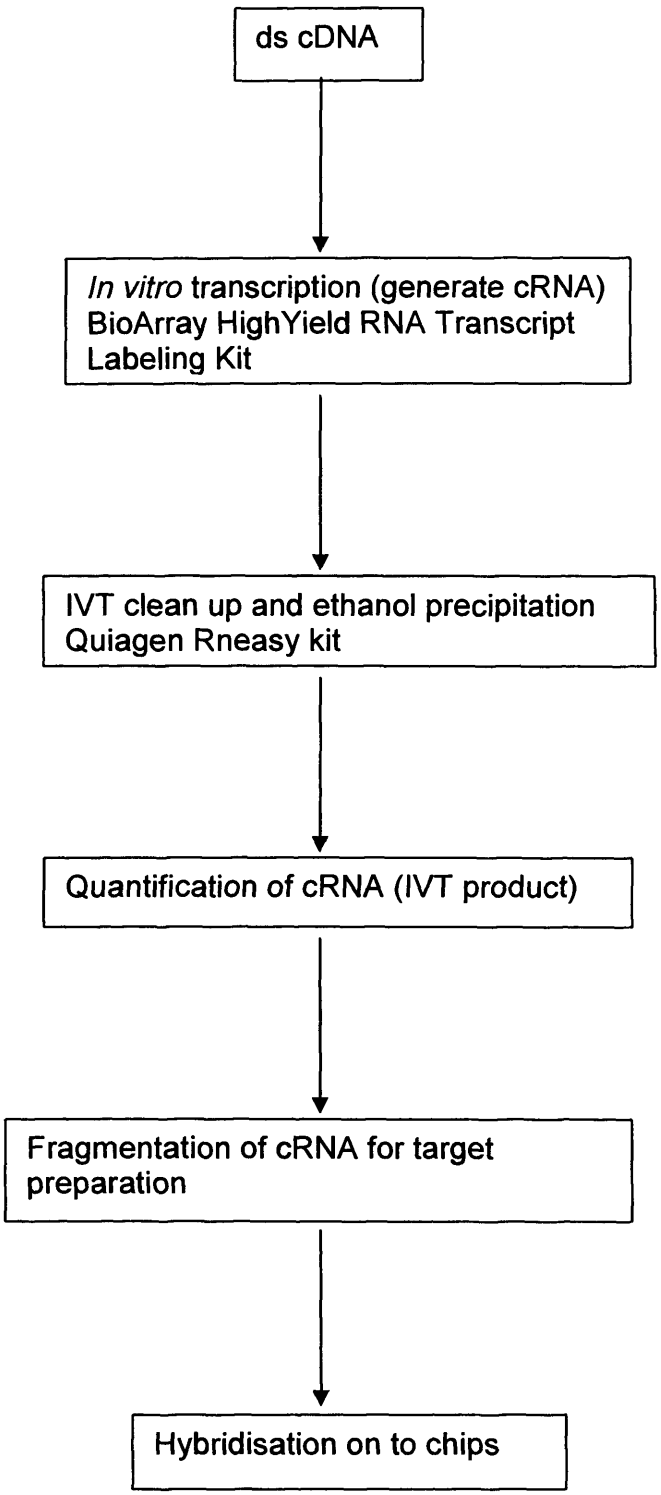
The four constituents were mixed together and the solution incubated at 37°C for 1 hour. The volumes were adjusted according to the amount of RNA used. The reaction was terminated by the addition of 0.1M EDTA pH 8.0 and 1mg/ml glycogen. The DNase enzyme was removed by adding an equal volume of phenol:chloroform:isoamylalcohol (25:24:1, Sigma, UK). The mixture was vortexed for 15 seconds, centrifuged at 14,000g for 10 minutes and the top aqueous layer transferred to a fresh tube. This was then repeated once with phenol:chloroform:isoamylalcohol and once with chloroform (Sigma, UK) to remove any remaining phenol. RNA was precipitated at -20°C for 1 hour with $1/5^{th}$ volume ammonium acetate (8M) and 3 volumes of 95% ethanol (-20°C). RNA was pelletted by centrifugation at 14,000g for 30 minutes at 4°C, washed with 200µl 70% ethanol (-20°C) and dried on the bench at 22°C (for about 10 minutes). RNA was resuspended at 5µg/µl and if not used immediately, stored at -80°C.

2.3 Oligonucleotide Microarray

2.3.1 Generation of Microarray Target

Summary of Process




ds cDNA
In vitro transcription (generate cRNA)
BioArray HighYield RNA Transcript
Labeling Kit
IVT clean up and ethanol precipitation
Quiagen Rneasy kit
Quantification of cRNA (IVT product)
Fragmentation of cRNA for target
preparation
Hybridisation on to chips

2.3.1.1 Synthesis of Double-Stranded cDNA From Total RNA

First Strand cDNA Synthesis

RNA used was at a concentration of 5µg/µl; 20µg (4 µl) RNA was used for each experiment.

i). Step 1: Primer Hybridisation

Reagents used:	DEPC water	5 µl
	RNA (20 µg)	4 µl
	T7- (dT) 24 primer (100pmol/ µl)	1 µl

T7-(dT)24 primer
5′ - GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-(dT)24 - 3′

Reagents were mixed in an eppendorf tube, incubated at 70°C for 10 minutes, briefly spun in the centrifuge to collect the condensation and placed on ice.

ii) Step 2: Temperature Adjustment

Reagents used:	5X First strand cDNA buffer	4 µl
	0.1M DTT	2 µl
	10mM dNTP mix	1 µl

The reagents were added to those used in step 1, and incubated at 42°C for 2 minutes.

iii) Step 3: First Strand Synthesis

Reagent used: SSII RT 3 µl

SSII RT was added to the reaction, mixed well, and incubated at 42°C for 1 hour. The final volume of the mixture was 20 µl.

Second Strand cDNA Synthesis

The First Strand reactions were placed on ice, then briefly centrifuged to bring down the condensation on the sides of the tubes. The Second Strand Final Reaction Composition listed below were added to the First Strand synthesis tube. The tube

was gently tapped to mix the reagents. The mixture was again briefly spun in a microcentrifuge to remove any further condensation and incubated at 16°C for 2 hours in a cooling waterbath. 2 µl (10U) T4 DNA Polymerase was added and the mixture incubated at 16°C for 5 minutes. 10 µl 0.5M EDTA was added. The mixture was then cleaned according to the clean-up protocol for cDNA, or stored at 20°C for later use.

Second Strand Final Reaction Composition

Component	Volume	Final concentration or amount in each
DEPC-treated water	90 µl	
5X Second Strand Reaction Buffer	30 µl	1X
10mM dATP,dCTP,DGTP, DTTP	3 µl	200 µM each
5 U/µl DNA ligase	2 µl	10 U
10 U/µl	4 µl	40 U
2 U/µl Rnase H	1 µl	2 U
Final volume	150 µl	

2.3.1.2 Clean-up of Double-Stranded cDNA

An equal volume of (25:24:1) phenol:chloroform:isoamylalcohol (saturated with 10 mM Tris-HCL pH 8.0/1mM EDTA, Sigma, UK) was added to the final cDNA synthesis preparation (162 µl) to a final volume of 324 µl. The mixture was vortexed for 15 seconds, centrifuged at 14,000g for 10 minutes and the top aqueous layer transferred to a fresh tube. This was repeated once with phenol:chloroform:isoamylalcohol and once with chloroform (Sigma, UK) to remove any remaining phenol. 0.5 volumes of ammonium acetate (7.5M) and 2.5 volumes of absolute ethanol (stored at -20°C) were added to the sample and vortexed. cDNA was pelleted by centrifugation at 14,000g for 20 minutes at 22°C, the supernatant carefully removed and the pellet washed with 500µl 80% ethanol (stored at -20°C). The mixture was again centrifuged at 14,000g for 5 minutes and the ethanol carefully removed. The ethanol wash was repeated once more, and the pellet airdried on the bench (about 15 minutes). The dried pellet was resuspended in 12µl Rnase-free water.

2.3.1.3 *In Vitro* Transcription

The ENZO BioArrayTM HIghYieldTM RNA Transcript Labelling Kit (T7) was used to generate large amounts of hybridisable biotin-labelled RNA targets by in vitro transcription from bacteriophage T7 RNA polymerase promoters. By using a T7 RNA polymerase and biotin-labelled nucleotides, large amounts of single stranded nonradioactive RNA molecules can be produced in vitro.

The following reagents were added in the order indicated in the table below to the cleaned-up final cDNA synthesis mixture at 22°C to avoid precipitation of DTT.

Reagent	Volume
Template DNA	12 µl
DEPC-treated water	10 µl
10X HY Reaction buffer	4 µl
10X Biotin Labelled Ribonucleotides	4 µl
10X DTT	4 µl
10X Rnase Inhibitor Mix	4 µl
20X T7 RNA Polymerase	2 µl
Total Volume	40 µl

The reagents were carefully mixed and the mixture collected at the bottom of the eppendorf by brief centrifugation. The tube was immediately placed in a 37°C H_2O bath for 5 hours. The contents of the tubes were carefully mixed every 30 minutes throughout the incubation. 5 hours is considered an optimum time for incubation; the longer the time, the greater the yield, although if left for too long, the increased chance of degradation by RNases. The RNA was then purified, or if not immediately used, was stored at -20°C.

2.3.1.4 Clean Up and Quantification of *In Vitro* Transcription Products

1 µl of unpurified IVT product was used for analysis using the bioanalyzer. The clean up step was essentially performed in order to remove unincorporated dNTPs so that the 260nm absorbance can determine the quantity of cRNA. One half of the IVT reaction was purified at a time. This was done firstly in case the sample was lost during purification and secondly because when IVT product yields are high, the amount of RNA in the whole reaction may exceed the capacity of the device used for

purification, thereby resulting in better overall yields.

i) Clean Up

The clean up procedure was performed using the RNeasy protocol (Qiagen). The reagent mixture was adjusted to 100 µl by the addition of DEPC-treated water. 350 µl Buffer RLT was added, then 250 µl 96% ethanol was added, and the reagents mixed thoroughly by pipetting. The sample (700 µl) was then applied to an RNeasy mini spin column placed in a 2ml collection tube. The sample was centrifuged at 14,000g for 15 seconds, and the flow-through discarded. The column was washed twice with 500µl buffer RPE. The first wash was centrifuged at 14,000g for 15 seconds, and the second wash for 2 minutes in order to dry the RNeasy silica-gel membrane. Total RNA was eluted by applying 50µl diethylene pyrocarbonate (DEPC)-treated water, which is Rnase-free, and centrifuging at 14,000g for 1 minute.

ii) Ethanol Precipitation

0.5 volumes of 7.5M ammonium acetate and 2.5 volumes of 100% ethanol (stored at -20°C) were added to the sample and vortexed for 15 seconds. The sample was precipitated for 1 hour at -20°C, after which the tube was centrifuged at 14,000g for 30 minutes at 4°C. The pellet was washed twice with 80% ethanol (-20°C). The pellet was airdried on the bench at room temperature before resuspension in 20µl DEPC-treated water.

iii) Quantification of the cRNA (IVT Product)

The concentration and purity of the RNA was checked using the Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA).

2.3.1.5 Fragmentation of the cRNA for Target Preparation

The following (Rnase-free) components were combined to a total volume of 20ml to make the fragmentation buffer and passed through a 0.2µm vacuum filter unit.

4.0ml 1M Tris acetate pH 8.1
0.64g magnesium acetate
0.98g potassium acetate
DEPC-treated water made up to 20ml

2µl of 5X fragmentation buffer were used for every 8µl of RNA plus water. The mixture was incubated at 94°C for 35 minutes, then put on ice. 1µl of fragmentation product was taken to check the concentration and purity using the Agilent Bioanalyzer.

2.3.1.6 Preparation of the Hybridisation Target

Reagents for the hybridisation target for the single probe array were prepared as shown below.

Component	Standard array	Final concentration
Fragmented RNA	15µg	0.05µg/µl
Control oligonucleotide B2	5µl	50pM
20X Eukaryotic hybridisation controls	15µl	100pM
Herring sperm DNA (10mg/ml)	3µl	0.1mg/ml
Acetylated BSA (50mg/ml)	3µl	0.5mg/ml
2X Hybridisation buffer	150µl	1X
DEPC-treated water	To final volume of 300µl	
Final volume	300µl	

The reagents were mixed together, heated to 99°C for 5 minutes, then to 45°C for 5 minutes. The hybridisation cocktail was then centrifuged at 14,000g for 5 minutes to remove any insoluble material. Meanwhile, the probe array cartridge was prepared by filling it with 250µl 1X Hybridisation Buffer through one of the septa. The probe array was incubated at 45°C for 10 minutes with rotation. The buffer solution was removed from the probe array cartridge and filled with 200µl of the clarified hybridisation cocktail avoiding any insoluble matter in the volume at the bottom of the tube. The probe array was placed in a rotisserie box in a 45°C oven at 60rpm. The probes were hybridised to the probe array during a 16-hour incubation. Figure 2.7 shows the appearance of the Affymetrix probe array.

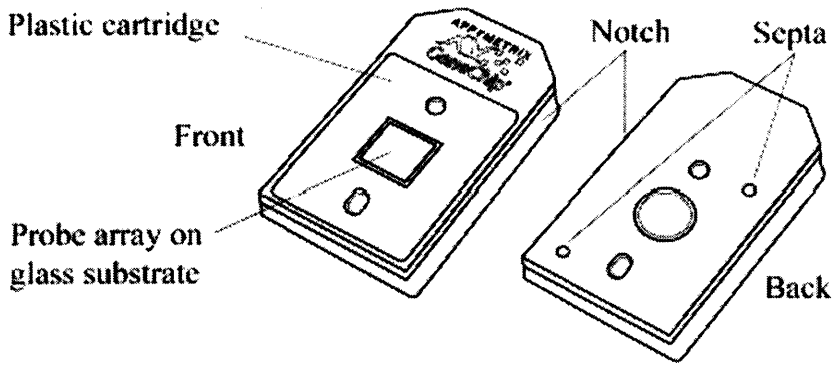


Figure 2.7. Affymetrix probe array
Immediately following hybridisation, the probe array was processed through an automated washing and staining protocol on the fluidics station. Wash buffer A (non-stringent) and wash buffer B (stringent) wee used for washing, and SAPE and antibody solutions were used for the staining. The components for these solutions are shown below.

Solutions Used

Non-Stringent Wash Buffer (Wash buffer A)
(6X SSPE, 0.01% Tween 20)

For 1000 ml:
300 ml of 20X SSPE
1.0 ml of 10% Tween-20
698 ml of water
Filter through a 0.2 µm filter

Stringent Wash Buffer (Wash buffer B)
(100 mM MES, 0.1 M [Na+], 0.01% Tween 20)

For 1000 ml:
83.3 ml of 12X MES Stock Buffer
5.2 ml of 5 M NaCl
1.0 ml of 10% Tween 20
910.5 ml of water
Filter through a 0.2 µm filter
Store at 2-8°C and shield from light

2X Stain Buffer
(Final 1X concentration: 100 mM MES, 1 M [Na+], 0.05% Tween 20)

For 250 ml:
41.7 ml 12X MES Stock Buffer 92.5 ml 5 M NaCl
2.5 ml 10% Tween 20
112.8 ml water
Filter through a 0.2 µm filter
Store at 2-8°C and shield from light

Streptavidin Phycoerythrin (SAPE) Stain Solution
(Total 1200µl)

600 µl 2X MES stain buffer
540 µl DEPC water
48 µl 50mg/ml acetylated BSA (final concentration 2 µg/µl)
12 µl of 1mg/ml SAPE (final concentration 10 µg/ml)

Antibody Solution
(Total 600µl)

300 µl of 2X MES stain buffer
266.4 µl DEPC water
24 µl of 50mg/ml acetylated BSA (final concentration 2 µg/ml)
6.0 µl of 10mg/ml normal goat IgG (final concentration 0.1 µg/ml)
3.6 µl of 0.5 mg/ml biotinylated antibody (final concentration 3 µg/ml)

Wash and Stain Protocol

Post Hyb Wash #1	10 cycles of 2 mixes/cycle with Wash Buffer A at 25°C
Post Hyb Wash #2	4 cycles of 15 mixes/cycle with Wash Buffer B at 50°C
Stain	Stain the probe array for 10 minutes in SAPE solution at 25°C
Post Stain Wash	10 cycles of 4 mixes/cycle with Wash Buffer A at 25°C
2nd Stain	Stain the probe array for 10 minutes in antibody solution at 25°C
3rd Stain	Stain the probe array for 10 minutes in SAPE solution at 25°C
Final Wash	15 cycles of 4 mixes/cycle with Wash Buffer A at 30°C. The holding temperature is 25°C

- Wash Buffer A = non-stringent wash buffer
- Wash Buffer B = stringent wash buffer

The hybridized probe array was stained with streptavidin phycoerythrin stain and antibody solutions, and scanned by the GeneArray® Scanner at the excitation

wavelength of 488 nm. The amount of light emitted at 570 nm is proportional to the bound target at each location on the probe array. Each probe array was scanned twice in order to improve the assay sensitivity and reduce background noise. The software calculates an average of the two images, defines the probe cells and computes an intensity for each cell. Each complete probe array image was stored in a separate data file and was saved with a data image file (.dat) extension.

2.4 Data Analysis

2.4.1 Expression Summary

Data analysis was performed in conjunction with Stephen Henderson, bioinformaticist at University College London. The basic principle behind analysing data from high density oligonucleotide arrays is converting the level of hybridisation of each gene into a numerical intensity value. Each gene is represented by 16-20 pairs of oligonucleotides, 25 base pairs in length, referred to as the probe set. Each pair of oligonucleotides comprises a perfect match (PM) and a mismatch (MM) probe, referred to as the probe pair. The mismatch probe differs from the PM probe by one central base pair, and this serves as a measure of non-specific binding (or background).

Perfect Match sequence: TACCGTTTAGGTAGGCTCCCATTTC
Mismatch sequence: TACCGTTTAGGTTGGCTCCCATTTC

Background subtraction, normalisation and expression values of our data were calculated using the rma algorithm [Irizarry et al., 2003], available as part of the Affymetrix package of the Bioconductor open-source software library for the statistical language R (http://www.bioconductor.org). Unlike the Affymetrix MAS5 algorithm normalisation of each experiment is carried out on all probe values prior to calculation of the expression summary for each probe-set rather than after. This is similar to the process of normalising the contrast and brightness of photographs or video images taken under different light levels. The images are simply quantile normalised to the chip with the median brightness. Figure 2.8 demonstrates the normalisation process. Again unlike MAS5, the PM values alone are used to calculate expression summary values for each probe-set. The rma algorithm was used due to its superior precision particularly for RNA transcripts of low abundance that is, the variability of repeated measurements is decreased. This has been assessed using spike-in experiments with known quantities of bacterial RNAs.

Differential expression between groups was calculated using a t-test and a step-

down False Discovery Rate (FDR) algorithm set to 0.05, implemented using the Bioconductor multtest package. This method controls the number of false positive expression difference calls that arise due to the vast degree of multiple testing of GEM. P-values from the t-test are adjusted upwards to make the level of all false positive results approximately 0.05.

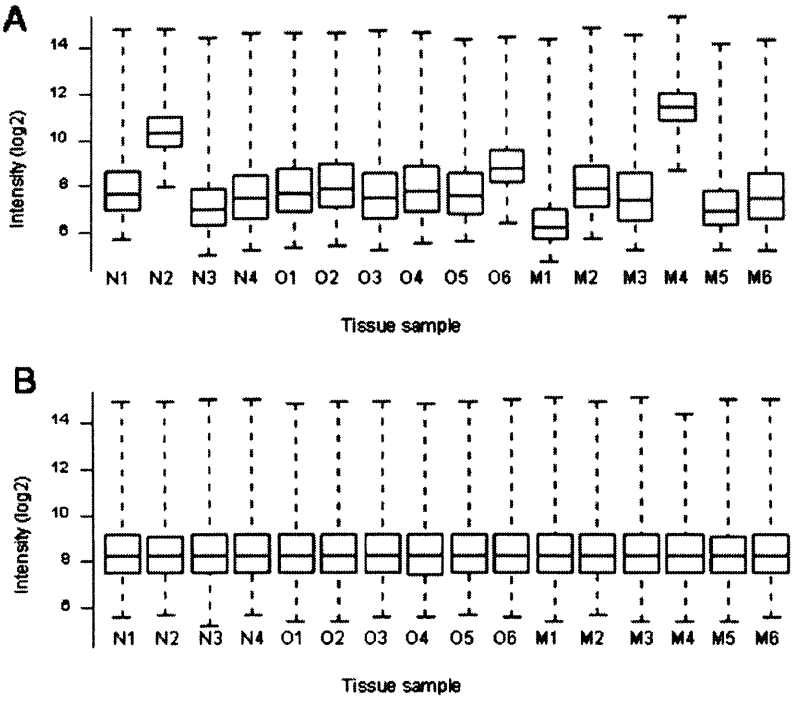


Figure 2.8. Boxplot showing image intensities of 16 chips before (A) and after (B) normalisation.
Box and whisker plots show a central median line, an interquartile box (25% and 75% range) and whiskers at 1.5 times the interquartile range. N=normal ovary, O=primary ovarian cancer, M=omental metastasis. **A** represents all 16 chips with image intensities before normalisation. N2 and M4 have high background intensities. **B** shows 16 chips normalised to each other, with the medians at the same value.

2.4.2 Average Linkage Hierarchical Clustering

Average linkage hierarchical clustering was performed using the Pearson correlation coefficient as a distance measure. Initially, each experiment is assigned to its own cluster, then the algorithm proceeds iteratively at each stage joining the two most similar clusters, continuing until there is just a single cluster. At each stage distances between clusters are recomputed to the average distance of the members of the clusters.

2.4.3 Comparative GEM Data

Publicly available GEM data from normal epithelial rich tissues and whole blood were obtained in CEL format from the Genomics Institute of the Novartis Foundation expression atlas (http://expression.gnf.org). Prostate and lung adenocarcinoma data were obtained in CEL format from the Whitehead Institute Centre for Genomic Research (http://www-genome.wi.mit.edu/cgi- bin/cancer/datasets.cgi). This was all analysed with our own data using rma (see section 2.4.1).

2.5 Real-Time Quantitative Reverse Transcriptase Polymerase Chain Reaction (QRT-PCR)

In order to confirm the results of the microarrays, 4 genes were selected for quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis.

2.5.1 General

Experiments involving the handling of nucleic acids were performed in accordance with standard practice [Sambrook and Gething, 1989]. Sterile glassware, tubes and disposable pipette tips were used and latex gloves were worn at all times. All aqueous solutions were either autoclaved (121°C, 20mins) or filtered through a 0.2 µm filter (Millipore Corp., Bedford, MA, USA).

2.5.2 Prevention of contamination

Precautions were taken to avoid false positives and to monitor the occurrence of cross-contamination. The specific measures taken were:

1. Separate rooms allocated for different sections of the optimisation experiment:
   RNA extractions
   PCR mixture preparation
   RNA sample dilutions
   cDNA loading
   Gel loading
   PCR run in the instrument

2. The inclusion of H_2O control tubes in all PCRs. H_2O was also used as a control to extract RNA from in parallel with the tested samples. Positive results with test samples were only accepted where all controls were negative.

3. Exposure of reaction components (H_2O, buffer) and instruments (Gilson pipettes, filtered pipette tips, microtubes) to ultra-violet light (UV 254nm) prior to the

addition of template. This was performed using a UV cross-linker (Stratagene Stratalinker 2400, 1 J/cm^2). Primer, probes and dNTP stock solutions were not exposed to UV light but were diluted in UV-treated H_2O or TE (Qiagen) and stored in aliquots at -20°C.

4. Use of designated sets of Gilson pipettes and 'filtered' pipette tips for setting up PCRs, for DNA loading, for post-PCR analysis and for handling plasmids containing cloned fragments.

5. Frequent changes of gloves and physical separation of solutions of target RNA/cDNA and from PCR products.

2.5.3 Instrumentation and Chemistry

The ABI PRISM® 7000 Sequence Detection System (ABI PRISM, Applera UK, Cheshire) was used in combination with SYBR® Green I dye chemistry.

2.5.4 Definitions Used in Real-Time PCR

The increase of the fluorescence is a direct consequence of the target amplification and is measured by the thermal cycler. Each signal is then divided by the fluorescence emitted by an internal reference dye (ROX), which is incorporated into the SYBR® Green I dye mix, in order to normalise for non-PCR related fluorescence fluctuations occurring well-to-well or over time.

The threshold cycle or C_T is the cycle at which a statistically significant increase in fluorescence is first detected.

The standard curve is a graph showing CT values plotted against the log of the initial amount of nucleic acid for a set of cDNA dilutions from a specific template and has been shown to be a straight line (Figure 2.9). In theory, the components of the PCR reaction are not limiting during the exponential phase of the amplification, therefore, the measurement of the fluorescence during this phase is proportional to the amount

of PCR product [Higuchi et al., 1993].

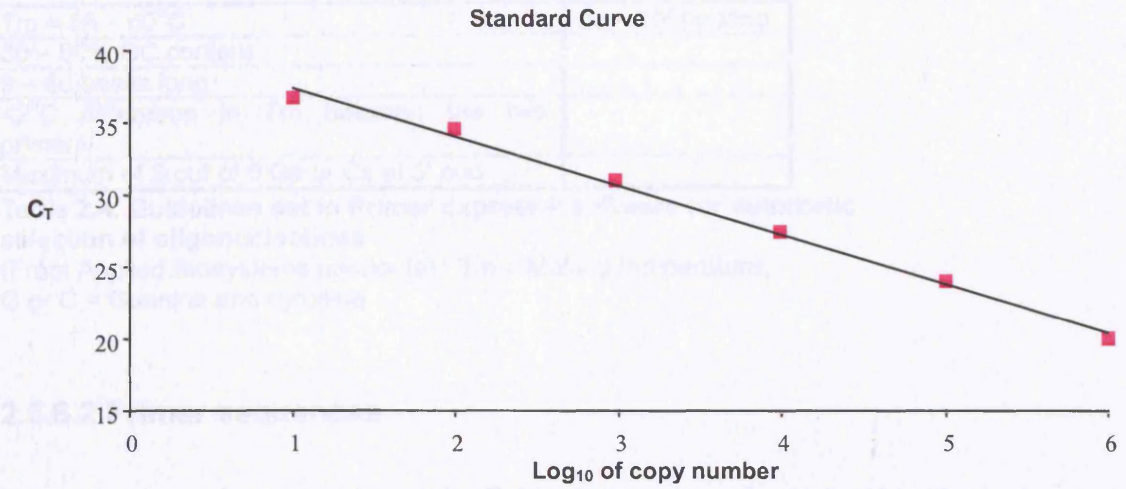


Figure 2.9. Standard curve graph.
Representative example of a standard curve showing the threshold cycle against the logarithm of the initial copy number in a set of standards

2.5.5 Genes Selected for QRT-PCR

Complementary DNA (cDNA) sequences from the encoding regions of hepsin, KLK6, SAA1 and the human glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH, endogenous control) were retrieved from the NCBI (GenBank, www.ncbi.nlm.nih.gov/BLAST) database and used as templates for the primer design.

2.5.6 Oligonucleotide design

2.5.6.1 Primer Express® Software

Primers (QIAGEN-OPERON, Cologne, Germany) were designed using Primer Express® software programme (PE Applied Biosystems, UK). The selection of primers is based on default parameters and guidelines (Table 2.4.). The target genes and GAPDH cDNA sequences were imported to the programme to be analysed. All primers have a similar annealing temperature. The amplicons are designed to be

between 50 and 150bp in length. This allows the amplification to be more efficient and it can be done in universal thermal cycling parameters.

Primers	Amplicon
Tm = 58 – 60°C	50 – 150bp long
30 – 80% GC content	
9 – 40 bases long	
<2°C difference in Tm between the two primers	
Maximum of 2 out of 5 Gs or Cs at 3' end	

Table 2.4. Guidelines set in Primer Express® software for automatic selection of oligonucleotides
(From Applied Biosystems handouts). Tm = Melting temperature, G or C = Guanine and cytosine

2.5.6.2 Primer sequences

The primer sequences shown in Table 2.5 were selected using the primer express software as mentioned in section 2.5.6.1 then re-entered into the NCBI (GenBank, www.ncbi.nlm.nih.gov/BLAST) database to confirm the specificity of the sequences.

Gene of Interest	Sense Primer	Antisense Primer	Product Size (bp)
MGB2	5'-CCGCTGCAGAGGCTATGG-3'	5'-CATCAGTCCAAAGTTTTTCAGAGTTCT-3'	86
HPN	5'-GGCTCGAGTCCCCATAATCAG-3'	5'-GGTAGCCAGCACAGAACATCTTG-3'	92
KLK6	5'-GCGGACCCTGCGACAAG-3'	5'-GGATAAGGACCCCACCACAGA-3'	84
SAA1	5'-TTCTCACGGGCCTGGTTTT-3'	5'-GCCTCGCCAAGGAACGA-3'	76
GAPDH	5'-GGAGTCAACGGATTTGGTCGTA-3'	5'GGCAACAATATCCACTTTACCAGAGT-3'	78

Table 2.5. Genes used for real time quantitative RT-PCR.
MGB2: mammaglobin 2, HPN: Hepsin or transmembrane protease, serine 1; TMPRSS1, KLK6: Kallikrein 6; Protease, serine, 9; PRSS9 or Protease M or neurosin, SAA1: Serum amyloid A1 or amyloid A, serum; SAA, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase.

Primers were further diluted in UV treated H_2O at the concentration of 10µM and 100µl aliquots were stored at -20°C.

2.5.7 Conventional PCR

[For principles of the PCR reaction, see section 2.6.4. The Polymerase Chain Reaction (PCR)]. The specificity of GAPDH and target gene primers was tested by a standard PCR amplification of template cDNA derived from a fetal library using the Expand High Fidelity PCR System (Roche, Germany). For a 25µl PCR reaction the conditions were as follows:

Constituent	Final Concentration
10 X Expand™ high fidelity buffer (15mM $MgCl_2$)	2.5µl (1 X)
dNTPs mix (1.25mM)	4µl (200µM)
Primers (10µM)	0.5µl (5µM each)
Taq High Fidelity	0.25µl (0.875Units)
cDNA (approx. 1ng/µl)	5µl
dH_2O	11.85µl

The reaction conditions were:

1 cycle	95°C	2mins
35 cycles	95°C	30secs
	60°C	30secs
	72°C	15secs
1 cycle	72°C	20secs

2.5.8 Gel Electrophoresis of Small Fragments

The GAPDH and target PCR product gene fragments were too small in size to be observed with a low percentage agarose gel. Therefore, a 4% agarose gel was prepared in 0.5 X TAE gel running buffer with 0.1µg/ml ethidium bromide (SIGMA). A 50bp DNA (GIBCO, Invitrogen) step ladder was included to estimate the size of the bands. Gels were captured by the Versadoc Imaging System (BioRad) for analysis.

2.5.9 Purification of cDNA fragments

Bands of the correct size were excised from the gel and cDNA was purified using the QIAquick gel extraction protocol (QIAGEN). The gel slice was weighted and 3 or 6 volumes (if the gel contained >2% agarose) of buffer QG (v/w) were added to the gel and incubated at 50oC till the gel was completely dissolved. One gel volume of isopropanol was added to the sample, mixed and applied to a QIAquick spin column. After 1min spin at 10,000g another 0.5ml of buffer QG was added, centrifuged and 0.75ml of buffer PE was applied to the column to wash for another minute. The cDNA was eluted at 20µl dH2O. The concentration of the cDNA products was measured using the Agilent 2100 bioanalyzer.

2.5.10 DNA Sequencing

The identity of the PCR products was verified by DNA sequencing using an automated application of the chain-termination method. The PCR purified product with the primers were given to our local PCR facility and the analysis was done using software (Beckman, CEQ 2000) supplied by the manufacturer (Beckman Coulter). Fifty to hundred fmoles of DNA template and 5pmoles/µl each of the primers were provided. The sequences were imported into BLAST as before to confirm the target genes.

2.5.11 QRT-PCR Consumables and Parameters

2.5.11.1 Consumables

The SYBR Green PCR Master Mix (4309155, Applied Biosystems) was used for every test. This kit is supplied at a 2X concentration. It contains SYBR Green 1 dye, AmpliTaq Gold DNA Polymerase, dNTPs with dUTP, Passive Reference 1 (ROX) and optimised buffer components. The reactions were transferred to MicroAmp® Optical 96-well Reaction Plates with Barcode (4306737, Applied Biosystems) and the plates were covered with ABI PRISM optical adhesive covers (4311971, Applied Biosystems) before they were transferred to the thermal cycler.

2.5.11.2 Parameters and PCR Conditions

The qPCR conditions used (primer concentration and amplification efficiencies), for the amplification of each cDNA sequence, were optimised individually for each gene. Primer optimisations were done in triplicate with triplicate non-template controls. Universal thermal cycling parameters were used for both genes as shown in Table 2.6. 1 µl of cDNA was used in a 25µl PCR mixture containing 1xSYBR® Green PCR mix (Applied Biosystems) and 0.3µM each primer for all genes apart from HPN where 0.6µM forward and reverse was used, and KLK6 where 0.9µM reverse primer was used. The threshold cycle (CT) which represents the PCR cycle at which an increase in reporter fluorescence above a baseline signal can first be detected, was calculated as previously described [Heid et al., 1996]. The relative expression of

each gene was determined on the basis of the CT value. The housekeeping gene GAPDH was used to normalise the quantity of cDNA used. Average GAPDH CT value was subtracted from that of each target gene to obtain a ΔCT value, i.e. normalise target gene expression relative to GAPDH. An average ΔCT value was obtained for each of the 5 groups of 19 cDNA ovarian samples (Normal: n=5, Low Malignant Potential (LMP): n=3, Primary: n=4 and Metastasis: n=2). Each average ΔCT was also subtracted from that of a calibrator (Average ΔCT value of all the Normal samples which provide the physiological expression of each gene target) to give the ΔΔCT value, i.e. normalised target gene expression in the different groups relative to Normal. Since CT values are measured when PCR amplification is still in the exponential phase then the relative quantitative value can be expressed as $2^{-\Delta\Delta CT}$ as 2 corresponds to the PCR product doubling in each cycle in the exponential phase.

Times And Temperatures			
Initial steps		**Each of 40 cycles**	
		Melting	**Annealing/Extension**
Hold	Hold*	Cycle	
10secs 50°C	10mins 95°C	15secs 95°C	1min 60°C

Table 2.6. Universal thermal cycling parameters for the qRT-PCR.
The 10mins hold at 95°C is required for the AmpliTaq Gold® DNA Polymerase activation

2.6 Semi-Quantitative Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) For Chemokine Analysis

As part of the search to identify factors which may be instrumental in metastasis of ovarian cancer, chemokines were investigated further by RT-PCR. The cDNA sequences for chemokines CXCR2, CXCR4, CCR1, CCR2, CCR3, CCR5, CCR7, CCR8 and CCR10 were obtained from the NCBI (GenBank, www.ncbi.nlm.nih.gov/BLAST) database.

2.6.1 Primer Design

The chemokine primers were designed according to the following principles:

1. The primers should be 20-26 base pairs in length, to ensure uniqueness within the template DNA.
2. The G+C content should be around 50%.
3. The primer should end with a G or C nucleotide to ensure strong annealing of the primer to the template for the initiation of extension by *Taq* polymerase.
4. Lengths of more than 3 of the same nucleotide should be avoided.
5. Sequences which could form secondary structures such as hairpins should be avoided.
6. A pair of primers used together for the amplification of a particular target sequence should be designed to have the same annealing temperature (T_a) of 50-60°C. This can be calculated using the following formula:

   T_a (C) = 4(G+C) + 2(A+T)

Chemokine	Sense Primer	Antisense Primer	Product size (bp)
CXCR2	5'-CTGCTGATCATGCTGTTCTGC-3'	5'-CTCACAGGTCTCCTGGATCAC-3'	160
CXCR4	5'-GACTATTCCCGACTTCATCTTTGCC-3'	5'-CAGATGAATGTCCACCTCGCTTTCC-3'	492
CCR1	5'-CGTGTTTGCCTTGCGGGCACGGACC-3'	5'-CTCATGGGTGAACAGGAAGTCTTG-3'	377
CCR2	5'-GGACTGCCTGAGACAAGCCACAAGC-3'	5'-GATGACTCTCACTGCCCTATGCCTC-3'	787
CCR3	5'-GGGAGAAGTGAAATGACAACCTCAC-3'	5'-TGCATGAGCAAGTGCCTGTGGAAGA-3'	954
CCR5	5'-GCACAGGGCTGTGAGGCTTATCTTC-3'	5'-GGTGTAAACTGAGCTTGCTCGCTCG-3'	305
CCR7	5'-GTATGCCTGTGTCAAGATGAGGTCAC-3'	5'-GTTGAGCAGGTAGGTATCGGTCATGG-3'	210
CCR8	5'-CCGCCATTATGGCTACCATCCCATTG-3'	5'-CCAACCTGATGGCCTTGGTCTTGTTG-3'	213
CCR10	5'-CAACGACGCTGTCGCCTCATCTTCC-3'	5'-GACATCCTTGCGTTTGCTGGCAGGG-3'	303

Table 2.7. Primers for chemokine genes

The primer sequences shown in Table 2.7 were selected using the primer express software as mentioned in section 2.5.6.1 then re-entered into the NCBI (GenBank, www.ncbi.nlm.nih.gov/BLAST) database to ensure that they only anneal to the desired sequence. The control housekeeping gene for this experiment was GAPDH Primers were ordered on the internet from MWG Biotech (Ebersberg, Germany), resuspended in 1ml distilled DEPC water and stored at -20°C. Each set of primers resulted in successful amplification of the correctly-sized amplicon from a suitable positive control.

2.6.2 Reverse Transcription: Basic Principles

Reverse transcription was performed using the SuperscriptTM first-strand synthesis system for RT-PCR (Invitrogen, Paisley, UK). Sample RNA, control RNA and Rnase-free water were used in this step in order to exclude DNA contamination. The essential steps are demonstrated in Figure 2.10.

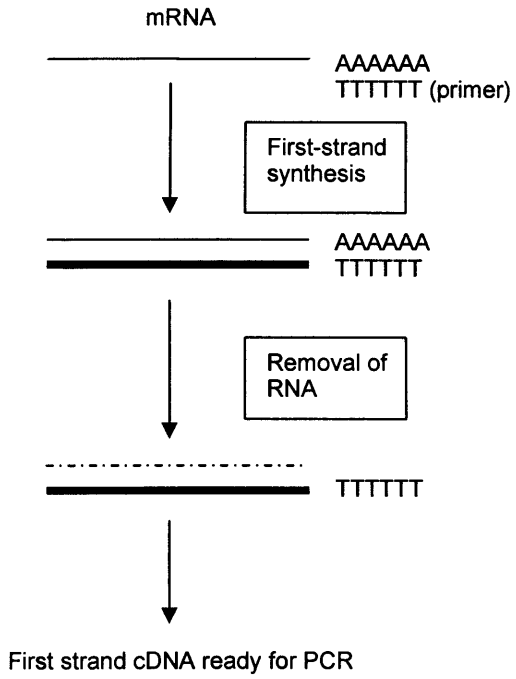


Figure 2.10. Summary of reverse transcription

RNA from 2 samples of normal ovary (N1 and N2) and 2 samples of primary ovarian cancer (O1 and O2) was purified as described in section 2.2.1 and contaminating DNA removed by treatment with DNase I (section 2.2.3). The reverse transcription step was performed on sample RNA, control RNA and distilled DEPC water to ensure all contaminating DNA was excluded.

2.6.3 Experimental Conditions

1 µl of oligo-dT primers (0.5 µg/ µl) were annealed to 1 µg of total RNA and 1 µl of 10mM dNTPs to a total volume of 10 µl by heating to 65°C for 5 minutes then chilling on ice for at least 1 minute. cDNA was synthesised by the addition of the following components:

RNA and primers	10 µl
10X RT buffer	2 µl
25mM $MgCl_2$	4 µl
0.1M Dithiothreitol (DTT)	2 µl
RnaseOUT	1 µl

This was incubated at 42°C for 2 minutes, then 1 µl of SuperScript II (50 U/ µl) added, and incubated for a further 50 minutes. The reaction was terminated by heating to 70°C for 15 minutes. The tube was chilled on ice, the reaction collected by brief centrifugation then 1 µl of Rnase H added to the tube and incubated at 37°C for 20 minutes. The resulting cDNA template was then amplified in the polymerase chain reaction.

2.6.4 The Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) was then performed in order to amplify the number of copies of the target cDNA sequence. This reaction consists of a chain reaction mediated by a DNA-dependent DNA polymerase. The essential steps are shown in Figure 2.11.

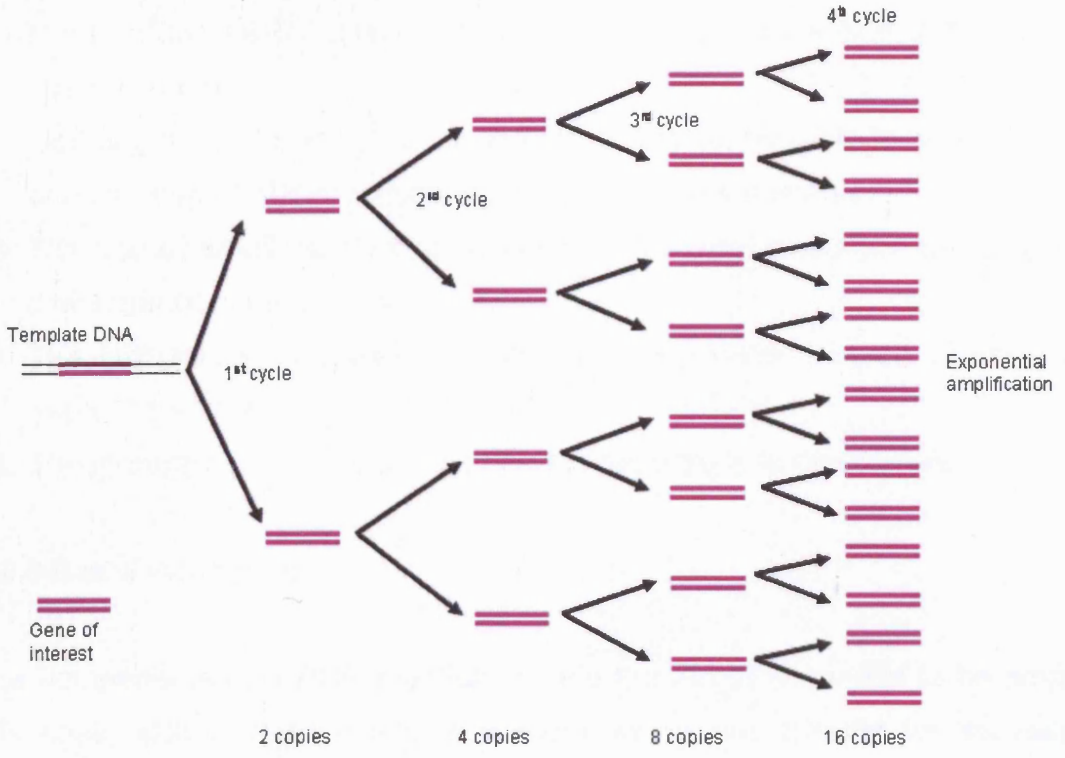


Figure 2.11. Basic steps of a PCR reaction.

2.6.5 Prevention of Contamination

PCR is used to amplify specific DNA sequences. Hence, it is susceptible to contamination by foreign DNA, from other objects or the atmosphere, resulting in the amplification of this DNA, rather than the experimental DNA. Therefore, the following strict precautions were taken to minimise this risk:

1. The use of a designated "PCR room" where no DNA is permitted. All components of the reaction mixture were added here; the target DNA was then added afterwards in a separate room.
2. All equipment and surfaces were cleaned with 100% ethanol prior to use.
3. A clean laboratory coat was worn, which remained in the PCR room at all times.
4. A clean, ultraviolet (UV)-irradiated containment hood with filtered air.
5. The use of autoclaved, double-distilled water.
6. The use of filter pipette tips to avoid aerosol contamination.

7. UV irradiation of all reaction tubes and water before setting up the PCR reaction.
8. Not touching anything with the pipette tip, except the inside of the reagent and reaction tubes.
9. Opening the reaction tubes for as short a time as possible to permit the addition of each reagent whilst preventing the entry of contaminants.
10. The use of small batches of samples so that any contamination affects only a small number of reactions.
11. The inclusion of negative controls containing water instead of DNA between every 3-4 samples.
12. The storage of reagents and pipette tips separately to other users.

2.6.6 Basic Principles

The components of a PCR reaction include the target sequence to be amplified, in this case, cDNA, oligonucleotide primers which are specific for the sequences flanking the target DNA fragment, buffer solution which contains salts to facilitate the polymerisation, DNA polymerase and dNTPs (deoxynucleotide triphosphates). Taq polymerase has been used in this study because it can resist temperatures above 96°C without becoming denatured, thereby being able to withstand the thermal cycling necessary to continuously denature and re-anneal the strands of synthesised DNA. A standard thermal cycling program consists of 30 cycles of amplification, and this typically results in the production of 230 copies of target DNA.

The PCR reaction works at different temperatures, depending upon the optimum temperature of each reaction. The first step is a denaturing step to break any double stranded DNA to single strands. Next, the temperature is lowered (50-60°C) so the primers can anneal to their complementary sites on the single-stranded genomic DNA. The temperature is then held at a short time for the Taq polymerase to work optimally (72°C), allowing the extension of the DNA chain from the annealed primers. The single-stranded DNA acts as a template on which the polymerase builds the complementary strand using the dNTP molecules. Once this extension step is completed, the temperature is raised to 96°C in order to denature the newly-formed double-stranded DNA, then the process repeats itself. This cyclical procedure results

in the exponential amplification of target DNA sequence situated between the primers.

2.6.7 PCR Amplification

PCR was performed using control for each pair of primers in a Primus 96 thermal cycler (MWG Biotech, Ebersberg, Germany) using the following constituents, with the Taq polymerase being added last:

Forward (sense) primer	2 µl
Reverse (antisense) primer	2 µl
10X buffer (with MgCl at	5 µl
10 mM dNT mix	1 µl
Distilled water	37.5 µl
Taq polymerase	0.5 µl
Final volume	48 µl

2 µl of cDNA was then added outside of the PCR room.

The reaction conditions for the chemokine primers were:

1. Lid heat at 110°C
2. 2 minutes at 94°C
3. 35 cycles of: 1 minute at 95°C
   1 minute 30 seconds at T_a°C
   1 minute 30 seconds at 72°C
4. 10 minutes at 72°C
5. Lid heat off
6. Hold at 4°C

The reaction conditions for GAPDH control primers were:

1. Lid heat at 110°C
2. 5 minutes at 95°C
3. 40 cycles of: 30 seconds at 95°C
   30 seconds at 50°C
   1 minute 30 seconds at 72°C

4. 20 minutes at 72°C
5. Lid heat off
6. Hold at 4°C

2.6.8 Visualisation Using Agarose Gel

When the amplification step was completed, 5 µl of each PCR reaction was separated by electrophoresis on a 1% agarose gel (agarose from Sigma, Saint Louis, Missouri, USA) in 0.5% Tris-acetate-EDTA (TAE) buffer. 10mg/ml ethidium bromide (Bio-Rad, California, USA) was used to stain the gel and detect DNA bands. 1 µl of 1-kb DNA ladder (GibcoBRL, Life Technologies, UK) was used as a size marker. The gels were viewed under ultra-violet light and photographed using a Kodak digital camcera 120. Figure 2.12 shows a typical gel of RT-PCR of sample RNA, RNA and water controls. Reverse transcription is performed for RNA where superscript is added, and where it is omitted. This is to control for DNA contamination. If DNA was found to be present, the sample was DNased (see section 2.2.3) and re-run.

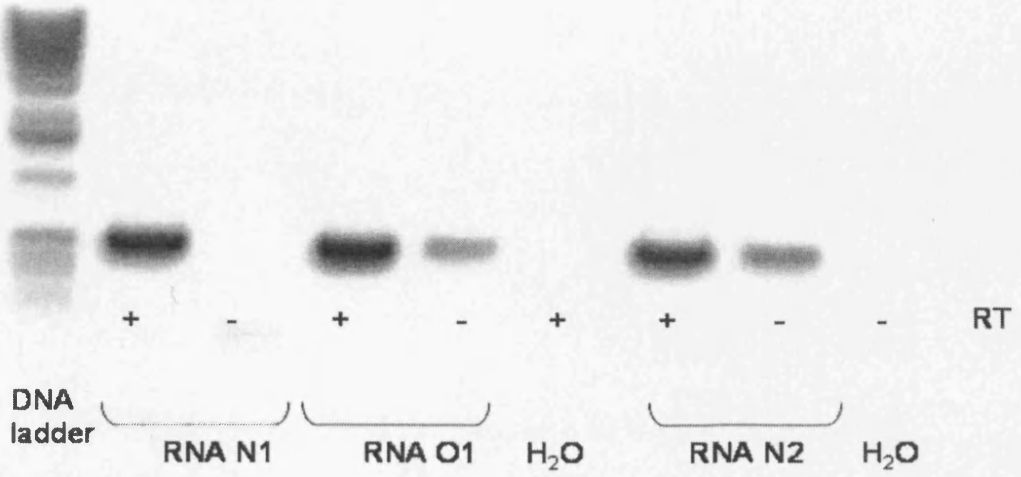
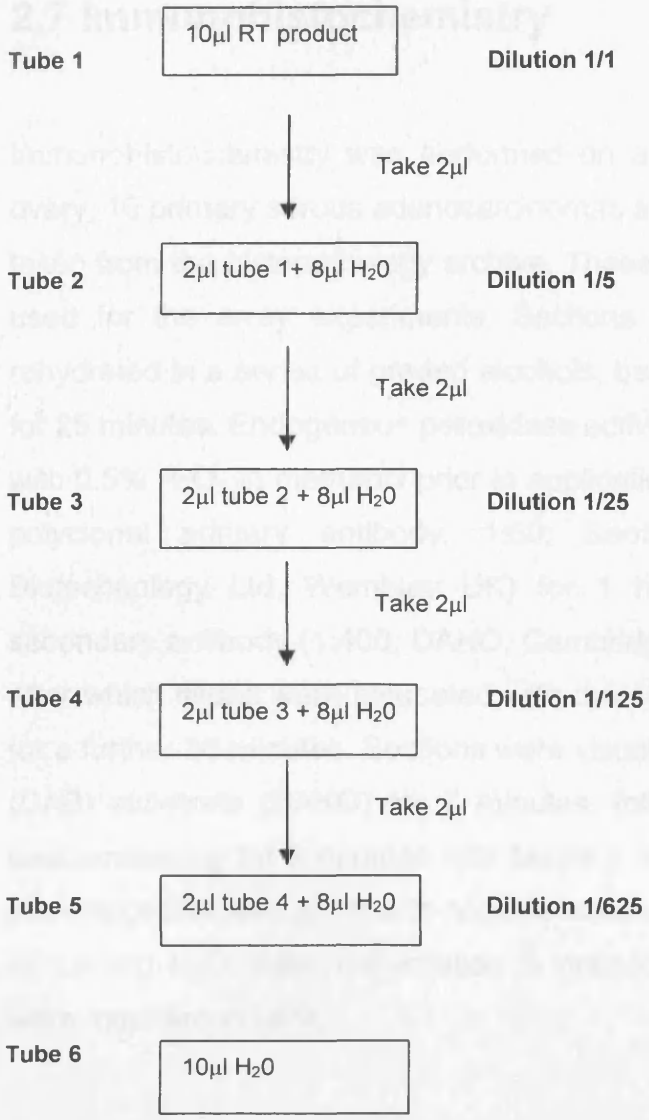


Figure 2.12 RT-PCR reaction.
RNAs N1, O1 and O2 are run with (+) and without (-) reverse transcriptase to check for DNA contamination. N1 = normal ovary, O1 & O2 = primary ovarian cancer. There is no gel band for N1 – RT signifying this is a clean sample. RNA O1 and O2 show bands when RT is not added, signifying there is DNA contamination in the RNA samples. The 2 H_2O controls check for general DNA contamination in the experiment. There are no bands visible, therefore the experiment was clean.

For the semi-quantitative RT-PCR, 10 µl of RT product were taken and sequential dilutions made so that each dilution was 1/5th of the previous reaction, down to a dilution of 1/625. Figure 2.13 shows a typical gel of a semi-quantitative RT-PCR of chemokines, with the intensity of the gel bands corresponding to serial dilutions.



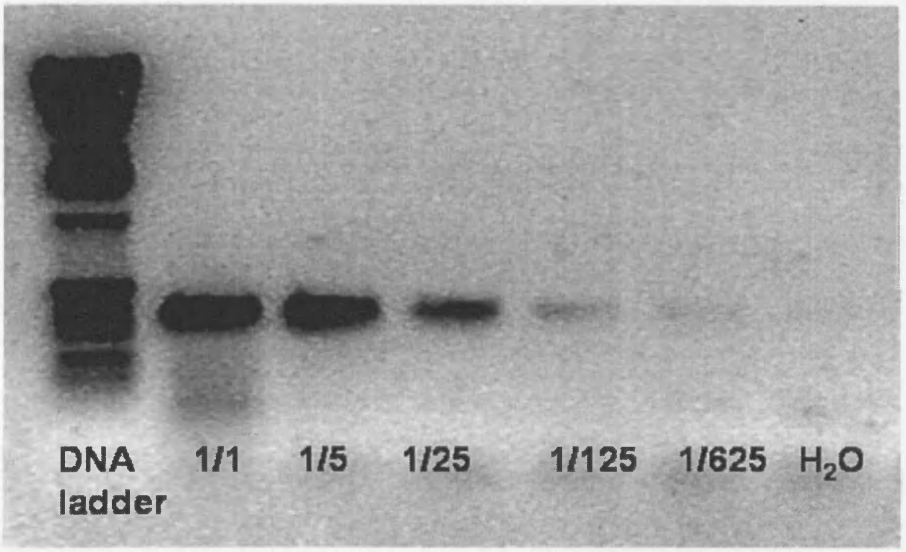


Figure 2.13 Serial dilutions of CCR5 chemokine in sample O2 (primary ovarian cancer).

2.7 Immunohistochemistry

Immunohistochemistry was performed on a further 30 paraffin blocks (10 normal ovary, 10 primary serous adenocarcinomas and 10 omental secondaries) which were taken from the histopathology archive. These patients are not related to the samples used for the array experiments. Sections were cut at 4µm, deparaffinised and rehydrated in a series of graded alcohols, before being heated in a microwave in TE for 25 minutes. Endogenous peroxidase activity was blocked by 10 minute incubation with 0.5% H_2O_2 in methanol prior to application of the primary antibody, hepsin (goat polyclonal primary antibody, 1:50; Santa Cruz Biotechnology Inc., Insight Biotechnology Ltd, Wembley UK) for 1 hour at 22C. A biotinylated, anti-goat secondary antibody (1:400; DAKO, Cambridgeshire UK) was applied for 30 minutes after which slides were incubated with the streptavidin-peroxidase complex (DAKO) for a further 30 minutes. Sections were visualized by application of diaminobenzidine (DAB) substrate (DAKO) for 7 minutes, followed by a wash in running H_2O and counterstaining for 2 minutes with Mayer's haematoxylin (DAKO). All sections were then dipped in acid alcohol to remove excess haematoxylin and immediately placed in running H_2O. After dehydration in graded alcohols, slides ended in xylene and were mounted in DPX.

CHAPTER 3

RESULTS

3.1 Clinical Material

Of the 47 clinical samples collected, 11 were diagnosed histopathologically as stage III primary serous cystadenocarcinomas of the ovary. Six of these were of adequate quality for analysis, as were the corresponding omental metastases from the same patients. The remaining 5 samples demonstrated degraded RNA. Four of the 8 normal ovarian samples were adequate for analysis and were included.

3.1.1 Microdissection

Sections of primary and secondary tumour were cut at 6μm thickness in the cryomicrotome for microdissection. The aim was to separate cancer cells from the underlying stromal cells in order to obtain a gene expression profile for the each subpopulation. Figure 3.1 shows the pre- and post-microdissection sections of one sample of primary serous adenocarcinoma. Microdissection was actually performed on non-stained tissue, but with the H&E sections adjacent in order to identify the relevant areas. H&E sections are used here for the purposes of demonstration.

Even though the tissue samples were placed immediately into RNAlater® (see section 2.1.3) in order to preserve the RNA, no RNA was subsequently obtained after RNA extraction. This could be due to two factors: an inadequate number of cells were retrieved or the microdissection process took too long, so the RNA had degraded by the time it was placed in the RNA preserving medium.

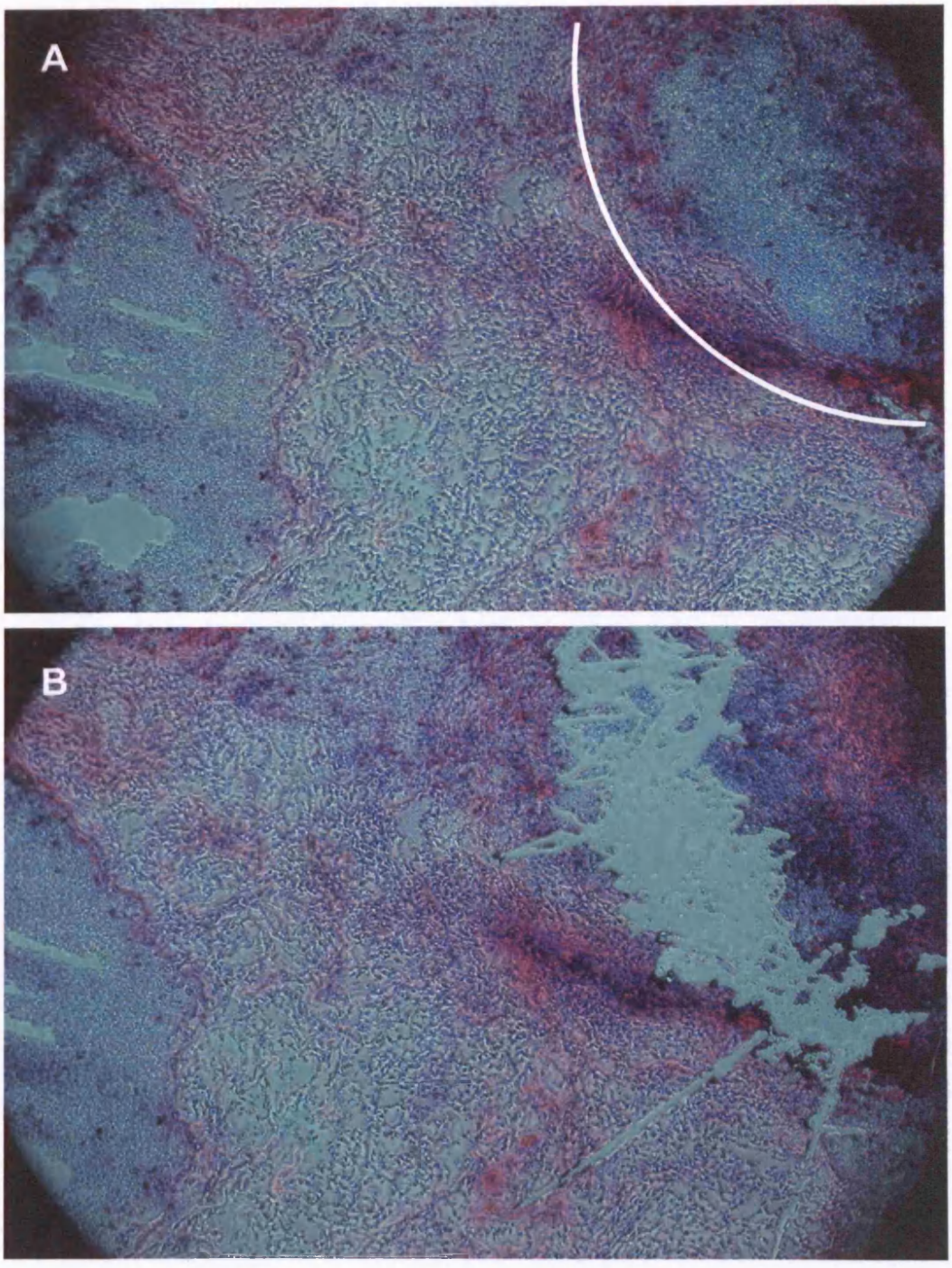


Figure 3.1. Microdissection
Slide A shows a pre-microdissection H&E staining of primary serous adenocarcinoma. The area at the top right of the picture bounded by the white line represents solid tumour. Slide B shows the post-microdissection view where tumour tissue has been removed.

Therefore the procedure was abandoned. Instead, block tissue was used for the cancer samples, and for the normal ovaries, epithelial tissue was macrodissected from the underlying stroma, in order to obtain a gene expression profile for mainly ovarian epithelium.

3.2 RNA Quality

The quality of the RNA extracted from the clinical samples was initially verified by gel electrophoresis and later by the Agilent 2100 bioanalyzer when it became available. Typical gels for gel electrophoresis are shown in Figure 3.2.

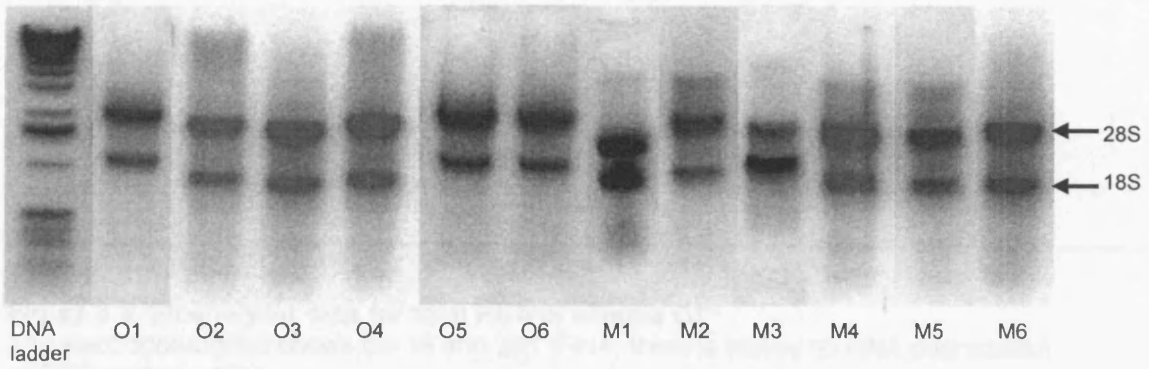


Figure 3.2. Purified total RNA
Total RNA was separated by 1% agarose gel electrophoresis. A composite of 12 samples (O1-6, and M1-6) is shown. The position of 28 and 18S ribosomal RNA is indicated.

The bioanalyzer presents the RNA data as an electropherogram (Figure 3.3) for each individual sample, with detailed data analysis (Figure 3.4), as a 12-well representation (Figure 3.5) in order to compare all samples to each other and as a simulation of the classic agarose gel (Figure 3.6).

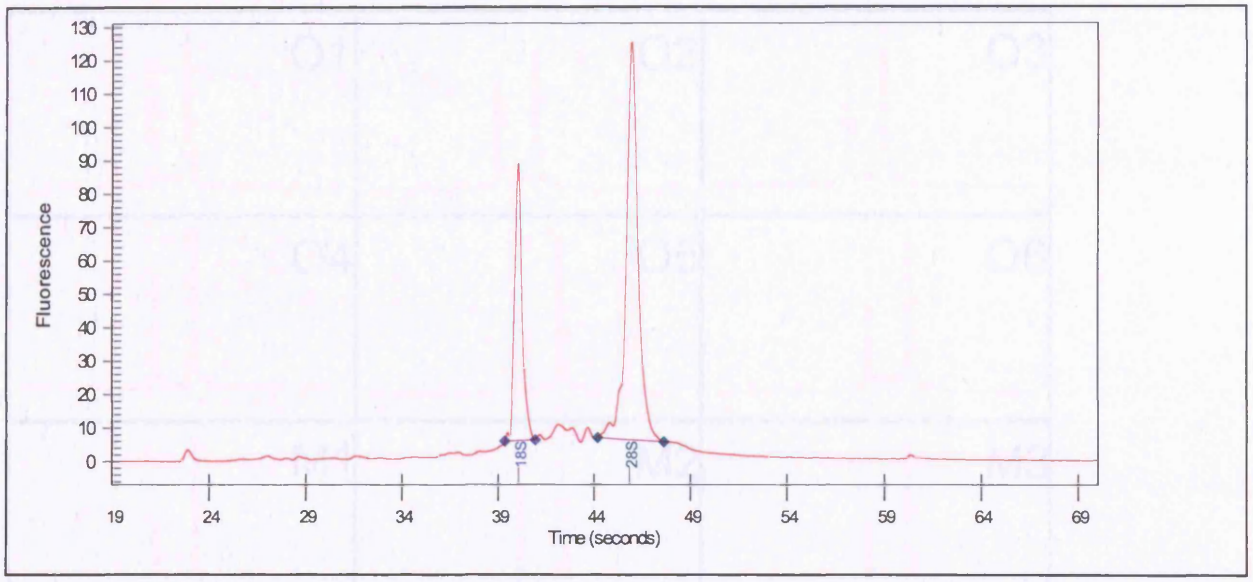


Figure 3.3. Bioanalyzer data for total RNA of sample O1
The electropherogram shows the 18 and 28S rRNA; there is clearly no RNA degradation or DNA contamination.

BioSizing_Total-RNA-Nano_00266_2002-03-18_14-39-35 O1

Fragment	Name	Start_Time(secs)	End_Time(secs)	Area	%_of_total_Area
1	18S	39.33	40.90	95.44	18.87
2	28S	44.17	47.60	183.08	36.21

RNA Area	505.67
RNA Concentration(ng/ul)	1,742.09
rRNA Ratio [28S / 18S]	1.92

Figure 3.4. Bioanalyzer data.
The bioanalyzer calculates the RNA concentration and the rRNA ratio, which in this case is 1.92. The ideal RNA ratio is 2.0.

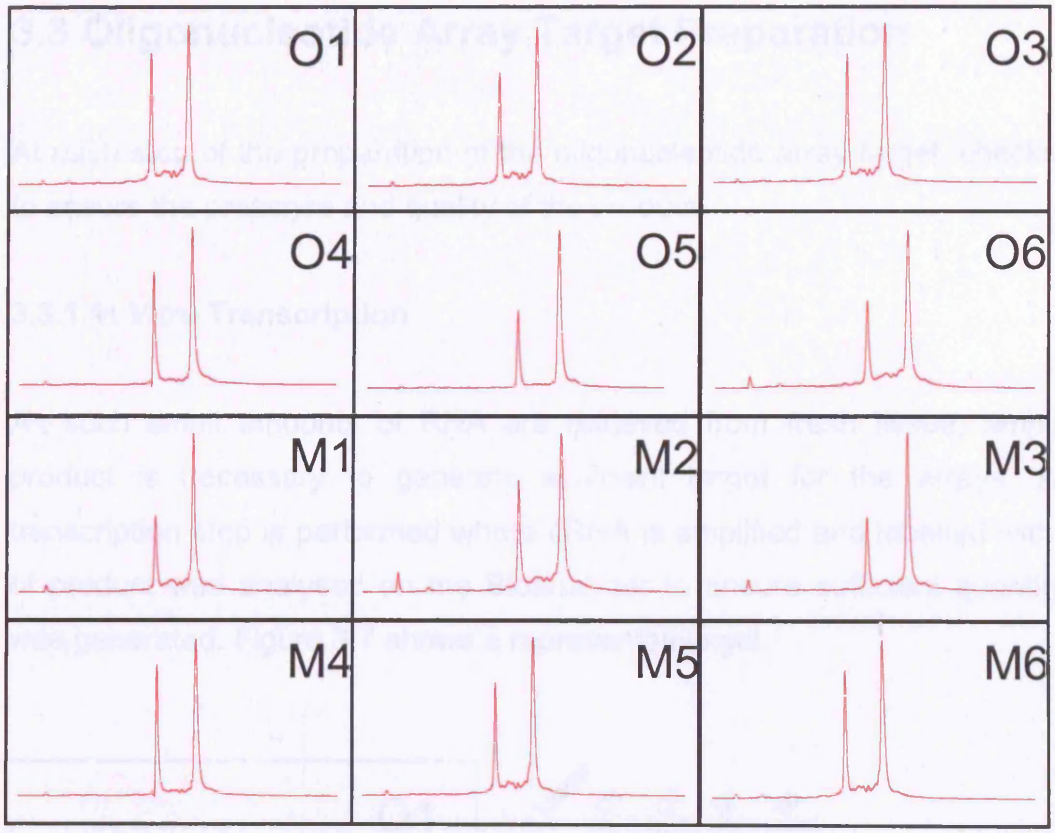


Figure 3.5. Electropherogram showing 12 well plate

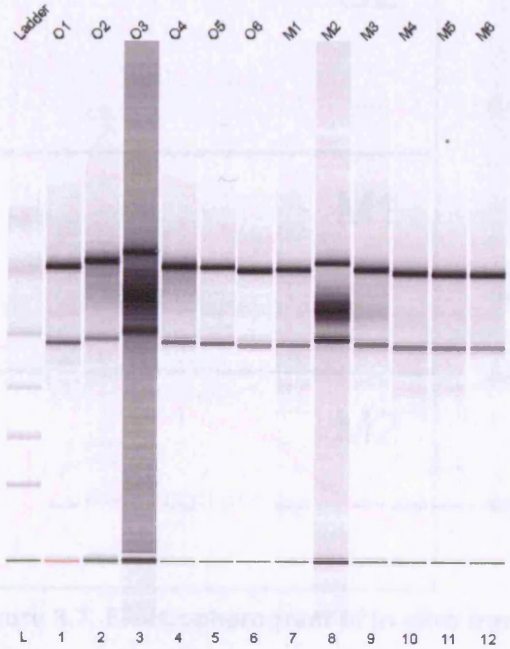


Figure 3.6. Electropherogram to resemble classic agarose gel

3.3 Oligonucleotide Array Target Preparation

At each step of the preparation of the oligonucleotide array target, checks are made to ensure the presence and quality of the product.

3.3.1 *In Vitro* Transcription

As such small amounts of RNA are retrieved from fresh tissue, amplification of product is necessary to generate sufficient target for the arrays. An in vitro transcription step is performed where cRNA is amplified and labelled with biotin. 1µl of product was analysed on the Bioanalyzer to ensure sufficient quantity of cRNA was generated. Figure 3.7 shows a representative gel.

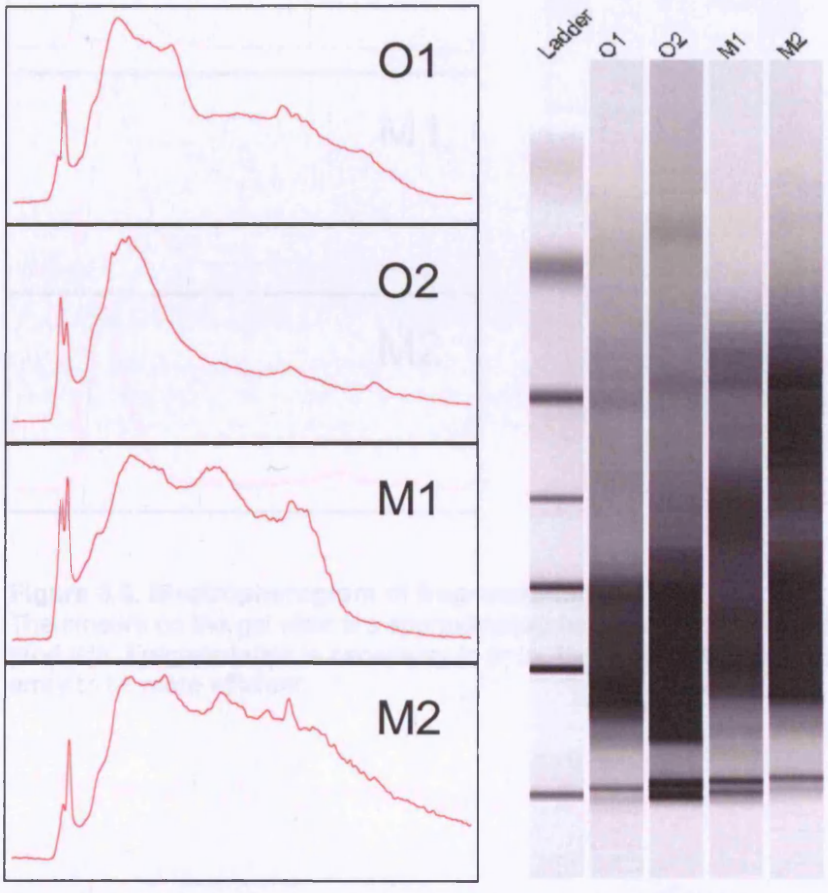


Figure 3.7. Electropherogram of in vitro transcription step

3.3.2 Fragmentation of cRNA

Following in vitro transcription, the products are cleaned up and precipitated in order to reduce the volume; they are then analysed using the Agilent Bioanalyzer (Figure 3.8).

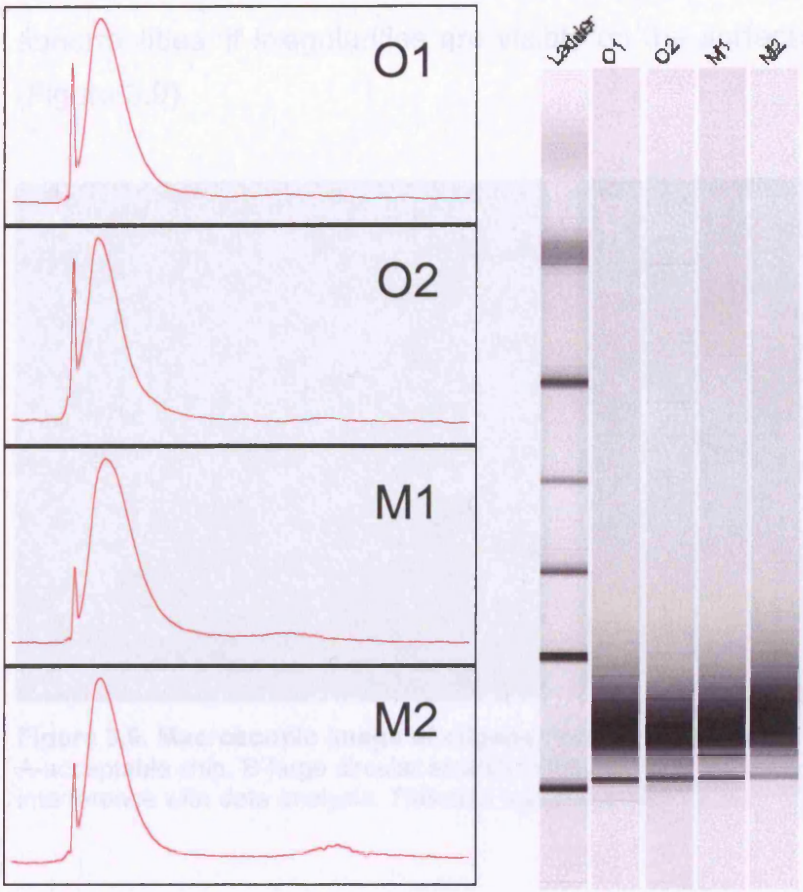


Figure 3.8. Electropherogram of fragmentation products.
The smears on the gel view are approximately half that of the post-IVT products. Fragmentation is necessary in order for hybridisation on to the array to be more efficient.

3.4 Scanning and Generation of Array Image

Hybridisation essentially allows the target oligonucleotides to bind to the relevant probes on the array. The amount of bound target gives a visual image of fluorescence. A large amount of target binding shows as a yellow dot; a low amount of binding shows as a black dot. The visual images are stored as dat files and converted into numerical cel files for analysis. Arrays are manually inspected for abnormalities; if irregularities are visible on the surface, then the array is discarded (Figure 3.9).

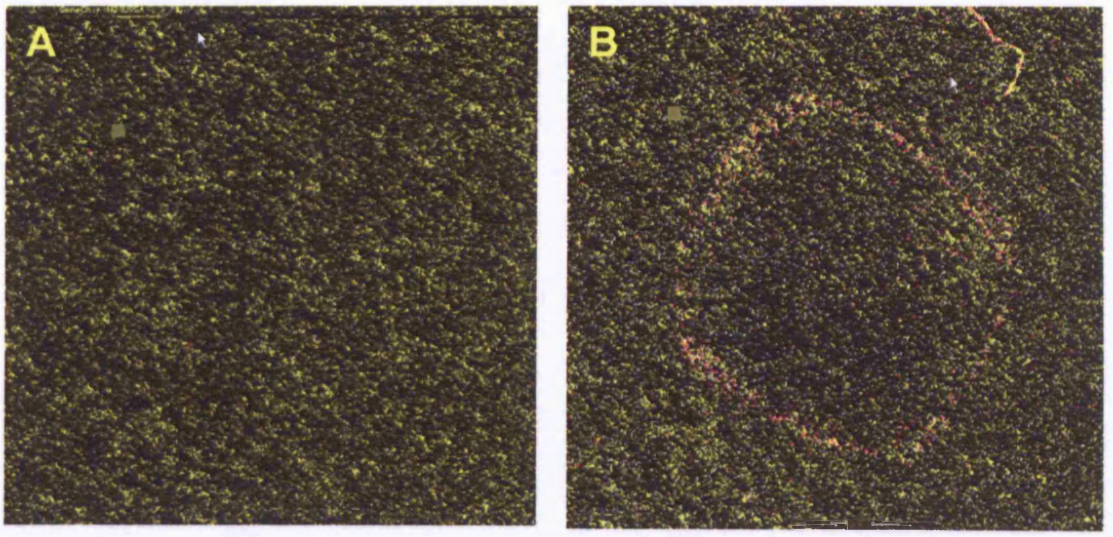


Figure 3.9. Macroscopic image of oligonucleotide arrays
A-acceptable chip. B-large circular scratch in the centre of the array, which would cause interference with data analysis. This chip was discarded.

3.5 GEM Profiling of Serous Ovarian Cancer: Primary Ovarian Disease

3.5.1 Hierarchical Clustering

Figure 3.10 shows a cluster dendogram (average linkage) of the 4 normal ovaries, 6 primary serous ovarian cancers and their corresponding omental metastases. There is clear separation between normal and cancer tissues. What is less clear is the clustering of primary and secondary cancer samples: there is no clear distinction between primary and omental tissue; in fact, sample pairs from the same patient are more likely to group together (O1/M1, O2/M2, O3/M3, O5/M5).

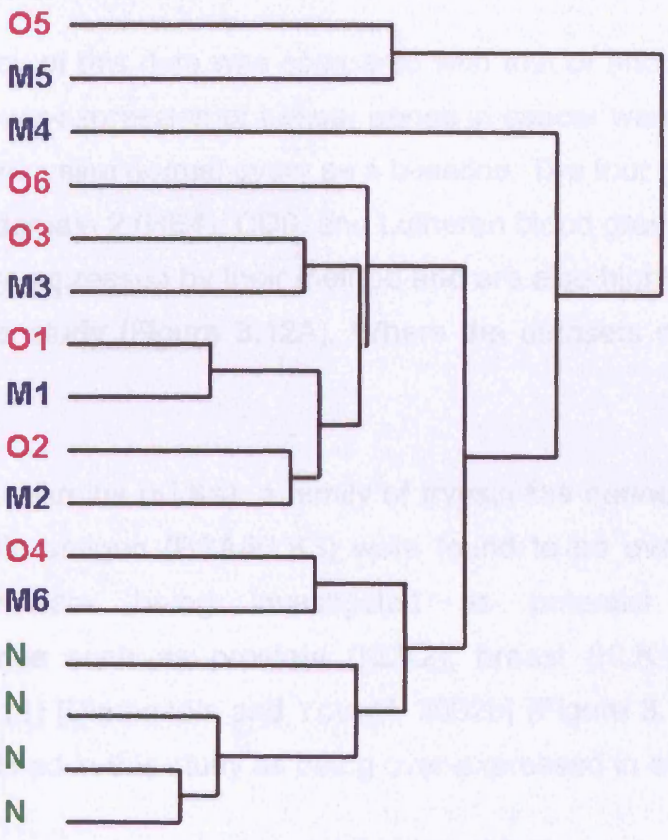


Figure 3.10. Cluster dendogram of normal and ovarian cancer samples.
Normal (N) ovarian tissue, primary ovarian cancer (P) and metastatic deposits (M).

3.5.2 Primary Ovarian Cancer Compared to Normal Ovarian Tissue

421 genes were more than 2-fold and 118 genes were more than 3-fold over-expressed in primary compared to normal tissue. Figure 3.11 shows significantly over-expressed genes in primary ovarian cancer sorted into functional groups. These groups include genes associated with epithelia and cell-cell contact such as secreted phosphoprotein 1 (OP), folate receptor 1, claudins-3 and 4 (CLDN3, 4), keratins-8, 18 and 19 (KRT8, 18, 19), and agrin (AGRN). These are also shown in Figure 3.12B and could reflect the epithelial origin of these tumours. Genes involved in cell division and growth include cyclin D1, cellular retinoic acid binding protein 2, and lipocalin 2 (oncogene 24p3). Metastasis and angiogenesis genes include jagged 2, tumour-associated calcium signal transducer 2 (TACSTD2), vascular endothelial growth factor (VEGF), CD24 antigen and neuromedin U.

The consistency of this data was compared with that of another study [Welsh et al., 2001] where over-expression of tumour genes in cancer were ranked according to a combined metric using normal ovary as a baseline. The four genes CD24, WAP four-disulfide core domain 2 (HE4), CD9, and Lutheran blood group (LU) were found to be the most highly expressed by their method and are also highly over-expressed in the data-set in this study (Figure 3.12A). Where the datasets overlap, they are highly consistent.

A number of kallikreins (KLKs), a family of trypsin-like serine proteases that include prostate-specific antigen (PSA/KLK3) were found to be over-expressed in ovarian cancer. KLKs are being investigated as potential serum markers for adenocarcinomas such as prostate (KLK2), breast (KLK10, 12, 13) and ovary (KLK6, 8, 10, 11) [Diamandis and Yousef, 2002b] (Figure 3.12C). In addition, KLK7 has been identified in this study as being over-expressed in ovarian cancer.

Table 3.1 lists the genes which are over-expressed in primary ovarian serous adenocarcinomas compared to normal ovarian tissue.

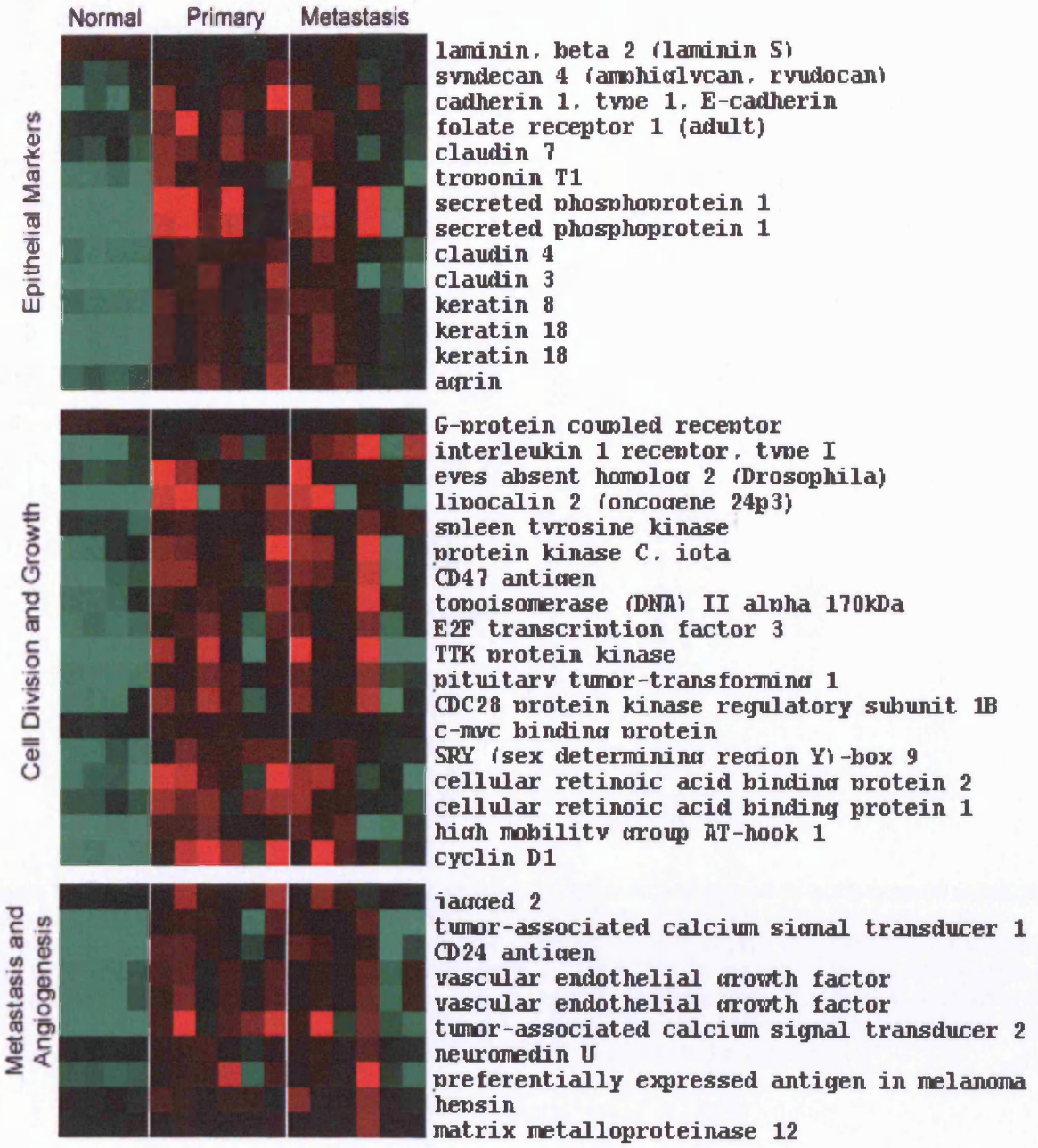


Figure 3.11. Heatmap showing genes up-regulated in serous ovarian primary and omental metastatic tumours compared to normal ovary.

A number of over-expressed genes previously associated with ovarian- and other cancers were identified, including VEGF, osteopontin (OP) [Kim et al., 2002a], preferentially expressed antigen in melanoma (PRAME) [Steinbach et al., 2002], TACSD2 (or GA733-1) [Shetye et al., 1989;Szala et al., 1990], and prostasin (PRSS8) [Mok et al., 2001a] (Figure 3.12D). These may all play a role in ovarian carcinogenesis.

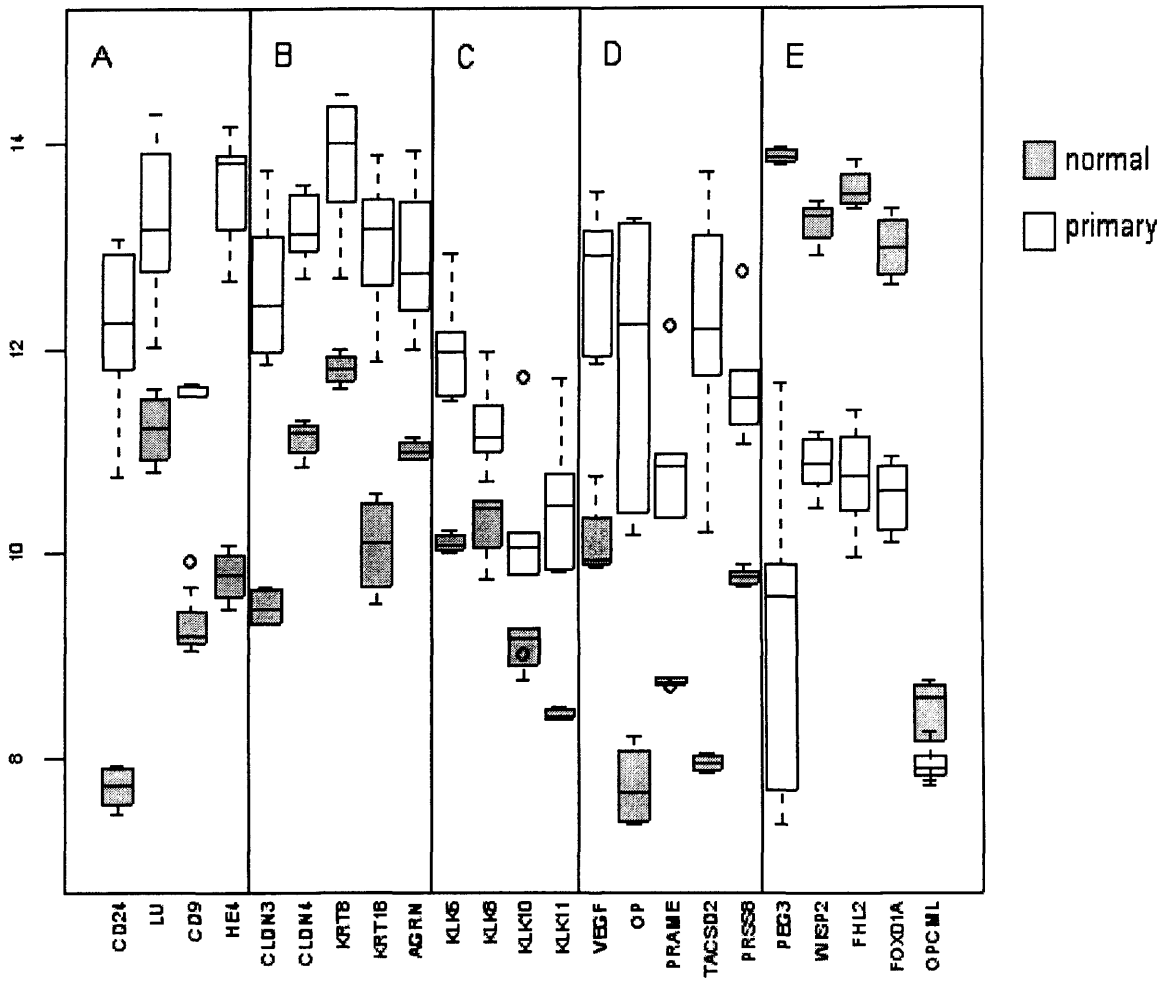


Figure 3.12. Box and whisker plots show expression of selected genes in both normal (shaded, n=4) and primary tissues (unshaded, n=6).
The selected genes are split into 5 categories (A-E) from left to right. (A) For comparison with previous ovarian cancer GEM studies, (B) Epithelial markers, (C) Kallikrein serine protease family, (D) A selection of previously described serous ovarian cancer markers and (E) Genes with loss of expression in primary tumours. Box and whisker plots show a central median line, an interquartile box, whiskers at 1.5 times the interquartile range, and outliers of these shown as circles.

172 genes were 3 fold down-regulated in primary ovarian cancer compared to normal ovary (Figure 3.13). Among these were putative tumour suppressors including the p53 mediator paternally expressed gene-3 (PEG-3) [Deng and Wu, 2000;Relaix et al., 1998], wnt-inducible signalling protein-2 (WISP-2) a member of the connective tissue growth factor family [Pennica et al., 1998], and the Rho-associated transcriptional coactivator four-and-a-half LIM domains 2 (FHL2) [Muller et al., 2002]. However, the recently reported putative tumour suppressor in ovarian cancer, opioid-binding protein (OPCML) did not appear to have any significant loss of expression in any of the samples studied here [Sellar et al., 2003] (Figure 3.12E).

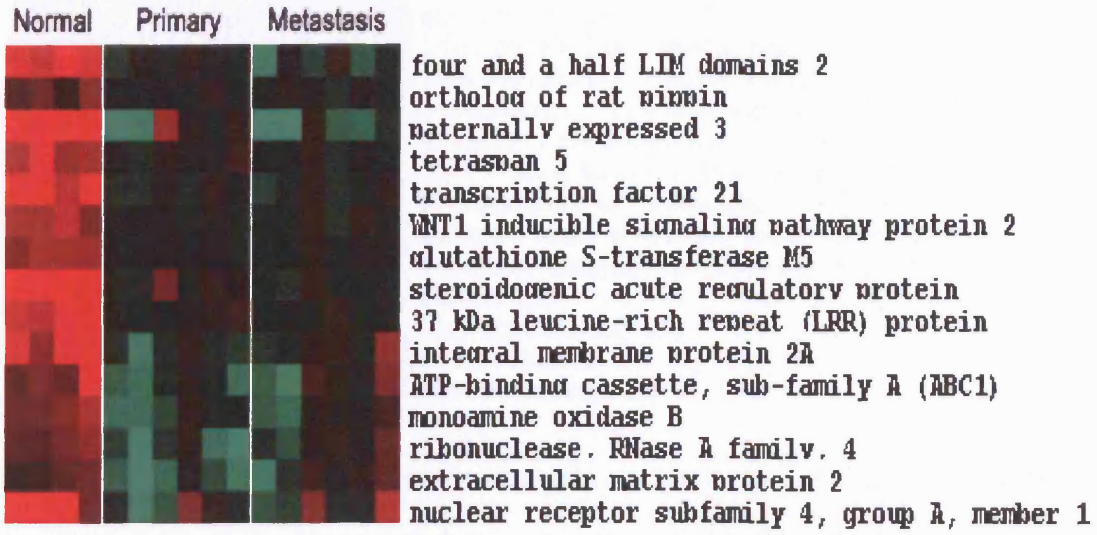


Figure 3.13. Genes down-regulated in primary and secondary serous ovarian cancer compared to normal ovary.
All differences are significant at the $p<0.05$ level after multiple testing adjustment (see methods).

3.5.3 Omental metastasis

Whilst there were 300 genes with more than 3-fold difference between normal and primary samples, there were only 35 equally large differences between primary and omental metastases, all greater in metastases (Figure 3.14). These genes fell into two main groups. Firstly, those that were over-expressed in just the omental samples compared to both primary ovarian cancers and normal ovarian tissue (Figure 3.15). These included serum amyloid A1 (SAA1), which is a marker of inflammation and immunoglobulin (Ig) lamda-locus which may reflect leucocyte infiltration. Many of the gene differences between primary and paired omental samples reflect the high adipocyte content in the omentum, such as adipsin, lipoprotein lipase, and perilipin. The second group comprised genes which were equally over-expressed in primary and secondary cancers compared to normal tissue. These included enhancer of zeste homolog 2 (EZH2) previously described as a predictor of metastasis in prostate [Varambally et al., 2002], and pituitary tumour-transforming 1 interacting protein (PTTG1) and Lamin B1 (LMNB1) predictors of metastasis in adenocarcinoma [Ramaswamy et al., 2003] (Figure 3.15). Table 3.2 shows genes over-expressed in Omental metastases compared to primary ovarian adenocarcinoma.

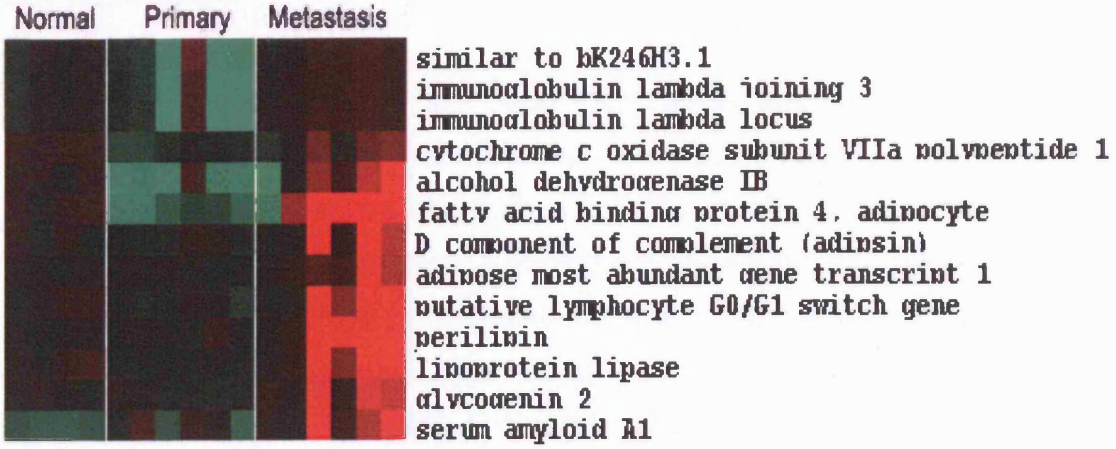


Figure 3.14. genes up-regulated in omental metastasis relative to normal ovary and primary ovarian cancer.
The predominance of genes associated with adipocytes reflects the omental background. All differences
are significant at the p<0.05 level after multiple testing adjustment (see methods).

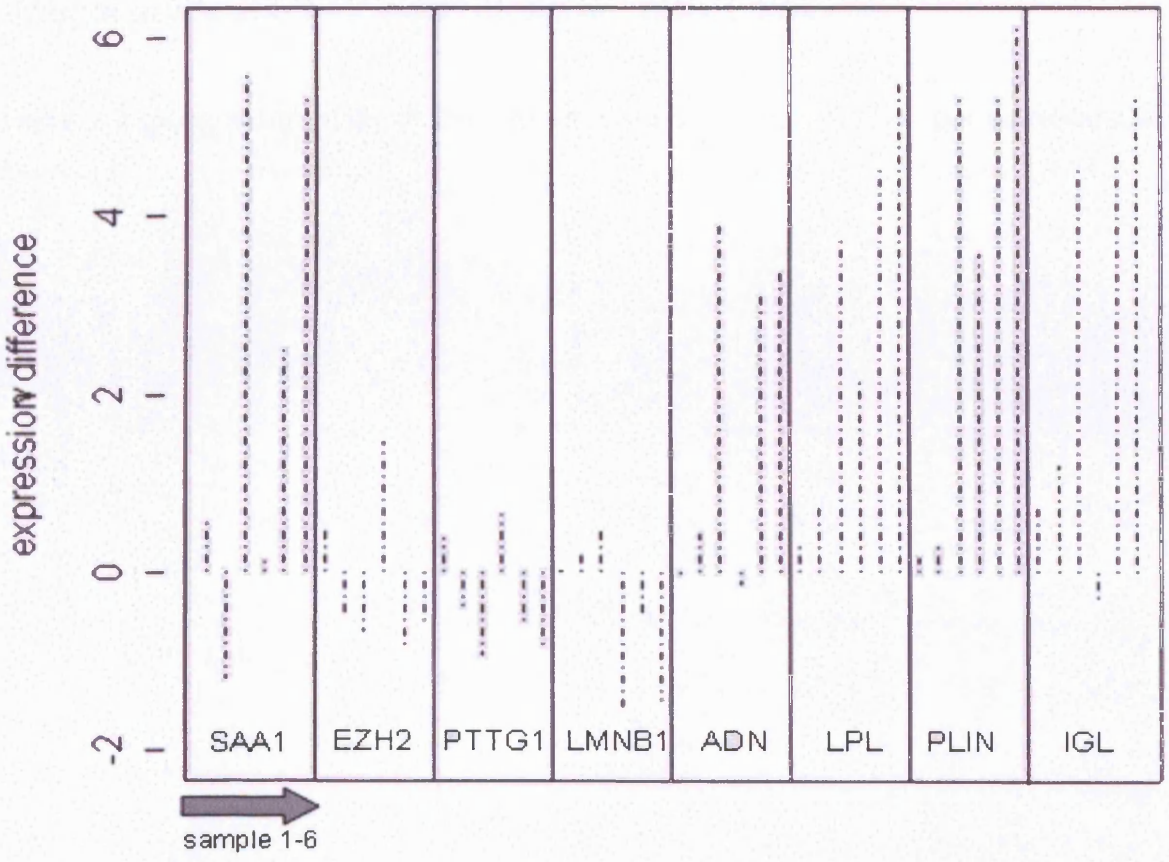


Figure 3.15. Expression of genes in metastatic and primary ovarian cancer samples (n=12, 6-paired).
The log difference of selected genes between the paired metastatic and primary ovarian cancer samples is plotted (metastatic – primary), so that upwards is higher in metastasis and downwards is lower. The paired p-values were SAA1- 0.03, EZH2-0.82, PTTG1-0.47, LMNB1-0.41, ADN-0.04, LPL-0.01, PLIN-0.01, IGL-0.01.

3.5.4 New biomarkers

A potential new biomarker MGB2 has been identified in this study which has (a) higher expression in both primary and metastatic samples compared to normal ovary, (b) high gross expression above the 80th percentile of all genes in primary and metastatic samples and (c) with high homology (58% amino acid identity) to the known serum marker MGB. Figure 15 shows the GEM profile of MGB2 compared to that of six other proteins that have been suggested as potential biomarkers: HPN [Tanimoto et al., 1997], IFI-15K, KLK6 [Diamandis and Yousef, 2002a], CP [Hough et al., 2001], SLPI [Shigemasa et al., 2001] and HE4 [Schummer et al., 1999] across a panel of epithelia rich tumours and tissues. This panel was comprised of publicly available Affymetrix data from: a) prostate adenocarcinoma [Singh et al., 2002] b) lung adenocarcinoma [Bhattacharjee et al., 2001] and c) the GNF gene expression atlas containing various primary epithelial tissues [Su et al., 2002] (see section 2.4.3). MGB2 in particular is very specific to ovarian adenocarcinoma.

Table 3.3 gives a summary of the names and abbreviations of all genes discussed in the text.

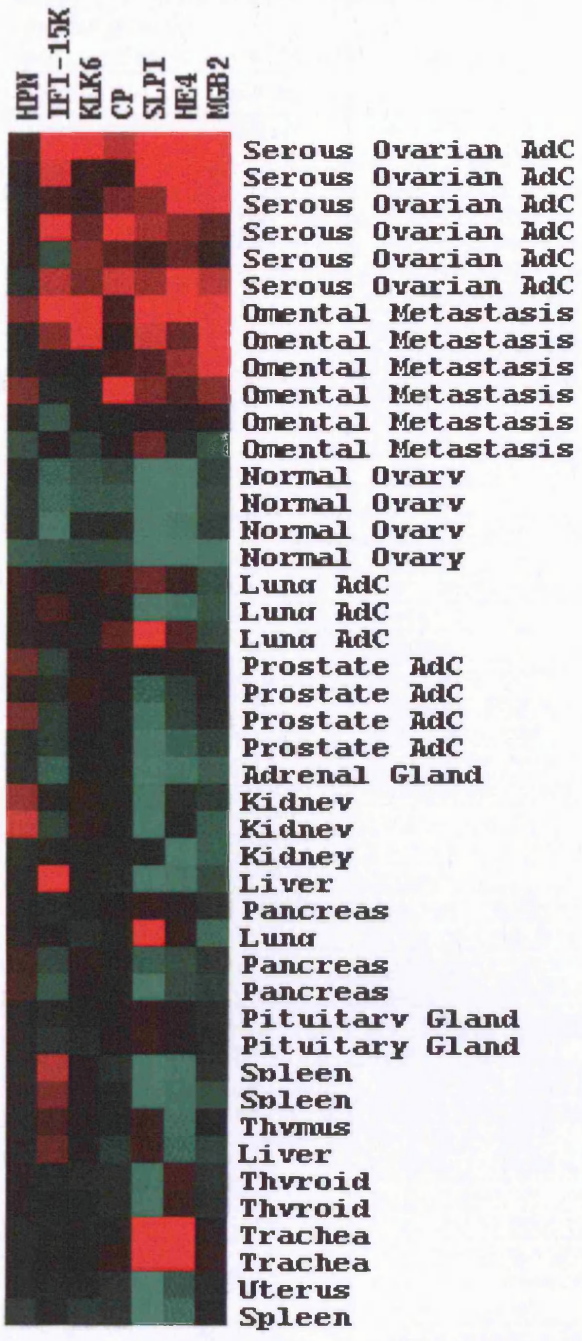


Figure 3.16. Gene expression profile of putative biomarker MGB2 in ovarian serous adenocarcinoma and a panel of other tissues. Comparison with six previously described biomarkers HPN, IFI-15K, KLK6, CP, SLPI and HE4. Serous ovarian AdC=primary serous ovarian adenocarcinoma, Omental Metastasis=serous ovarian omental metastasis, Lung AdC=lung adenocarcinoma, Prostate AdC=prostate adenocarcinoma. Adrenal gland, Kidney, Liver, Pancreas, Pituitary Gland, Lung, Spleen, Thymus, Thyroid, Trachea and Uterus all represent corresponding normal tissue specimens.

Table 3.1. Genes over-expressed in primary ovarian serous adenocarcinomas compared to normal ovary.

Average difference and p values are shown.

Probe set	Acc no.	Gene description	P-value	Avg. diff.
34342_s_at	AF052124	secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)	0.00005	4.74559
266_s_at	L33930	CD24 antigen (small cell lung carcinoma cluster 4 antigen)	0.00002	4.47336
291_s_at	J04152	tumor-associated calcium signal transducer 2	0.00035	4.23852
2092_s_at	J04765	secreted phosphoprotein 1 (osteopontin, bone sialoprotein I, early T-lymphocyte activation 1)	0.00049	4.19776
32275_at	X04470	secretory leukocyte protease inhibitor (antileukoproteinase)	0.00001	4.14516
575_s_at	M93036	tumor-associated calcium signal transducer 1	0.00010	3.94321
33933_at	X63187	WAP four-disulfide core domain 2	0.00000	3.81258
41066_at	AF071219	secretoglobin, family 2A, member 1	0.00111	3.48639
36133_at	AL031058	desmoplakin	0.00116	3.44008
700_s_at	X52229	mucin 1, transmembrane	0.00098	3.33690
668_s_at	L22524	matrix metalloproteinase 7 (matrilysin, uterine)	0.00906	3.21742
41294_at	AJ238246	keratin 7	0.00378	3.17625
38784_g_at	J05581	mucin 1, transmembrane	0.00129	3.16934
33904_at	AB000714	claudin 3	0.00006	3.10898
38324_at	AD000684	liver-specific bHLH-Zip transcription factor	0.00051	2.98481
35766_at	M26326	keratin 18	0.00006	2.96373
36861_at	AL049946	adlican	0.00030	2.91318
35822_at	L15702	B-factor, properdin	0.00558	2.76628
37534_at	Y07593	coxsackie virus and adenovirus receptor	0.00000	2.71204
35995_at	AF067656	ZW10 interactor	0.00133	2.70696
38783_at	J05581	mucin 1, transmembrane	0.00064	2.63835
36100_at	AF022375	vascular endothelial growth factor	0.00007	2.59976
32821_at	AI762213	lipocalin 2 (oncogene 24p3)	0.00710	2.54362
39008_at	M13699	ceruloplasmin (ferroxidase)	0.00014	2.46470
31792_at	M20560	annexin A3	0.00312	2.42945
1953_at	AF024710	vascular endothelial growth factor	0.00029	2.42295
38631_at	M92357	tumor necrosis factor, alpha-induced protein 2	0.00143	2.39254
33232_at	AI017574	cysteine-rich protein 1 (intestinal)	0.00638	2.38955
1107_s_at	M13755	interferon, alpha-inducible protein (clone IFI-15K)	0.00546	2.37402
927_s_at	J05582	mucin 1, transmembrane	0.00138	2.35302
2017_s_at	M64349	cyclin D1 (PRAD1	0.00246	2.34518
39704_s_at	L17131	high mobility group AT-hook 1	0.00001	2.29549
32715_at	N90862	vesicle-associated membrane protein 8 (endobrevin)	0.00073	2.27559
40401_at	AL050069	docking protein 5	0.00105	2.27378
40690_at	X54942	CDC28 protein kinase regulatory subunit 2	0.01344	2.27151
35207_at	X76180	sodium channel, nonvoltage-gated 1 alpha	0.00031	2.26287
35937_at	U65416	MHC class I polypeptide-related sequence B	0.00486	2.20814
41377_f_at	J05428	UDP glycosyltransferase 2 family, polypeptide B7	0.01159	2.17544
1882_g_at	D43969	runt-related transcription factor 1 (acute myeloid leukemia 1; aml1 oncogene)	0.00458	2.16153
1057_at	M97815	cellular retinoic acid binding protein 2	0.00283	2.16073
32072_at	U40434	mesothelin	0.00284	2.15042
36869_at	X69699	paired box gene 8	0.00004	2.14389
40074_at	X16396	methylene tetrahydrofolate dehydrogenase (NAD+ dependent), methenyltetrahydrofolate cyclohydrolase	0.00172	2.13215
38418_at	X59798	cyclin D1 (PRAD1	0.00305	2.13167
37892_at	J04177	collagen, type XI, alpha 1	0.05986	2.11769
37558_at	U97188	IGF-II mRNA-binding protein 3	0.06410	2.10125
40035_at	AB012917	kallikrein 11	0.00072	2.07794
572_at	M86699	TTK protein kinase	0.00483	2.06860
36197_at	Y08374	chitinase 3-like 1 (cartilage glycoprotein-39)	0.00134	2.06780
977_s_at	Z35402	cadherin 1, type 1, E-cadherin (epithelial)	0.00212	2.06536
39389_at	M38690	CD9 antigen (p24)	0.00033	2.06004
35276_at	AB000712	claudin 4	0.00000	2.03504
33824_at	X74929	keratin 8	0.00043	2.02756

1585_at	M34309	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)	0.00424	2.02086
1651_at	U73379	ubiquitin-conjugating enzyme E2C	0.00607	2.00288
40093_at	X83425	Lutheran blood group (Auberger b antigen included)	0.00104	1.99645
37591_at	U94592	uncoupling protein 2 (mitochondrial, proton carrier)	0.01026	1.97942
35127_at	AI039144	histone 1, H2ae	0.00745	1.93914
37554_at	U62801	kallikrein 6 (neurosin, zyme)	0.00021	1.91208
157_at	U64871	preferentially expressed antigen in melanoma	0.00943	1.90772
38749_at	AI936826	G protein-coupled receptor 39	0.00062	1.89624
38482_at	AJ011497	claudin 7	0.00013	1.89609
31888_s_at	AF001294	pleckstrin homology-like domain, family A, member 2	0.00471	1.88840
33338_at	M97936	signal transducer and activator of transcription 1, 91kDa	0.00164	1.87689
634_at	L41351	protease, serine, 8 (prostasin)	0.00047	1.87432
33454_at	AF016903	agrin	0.00111	1.87267
34213_at	AB020676	KIBRA protein	0.00002	1.86377
149_at	U88629	DEAD (Asp-Glu-Ala-Asp) box polypeptide 39	0.00177	1.84815
41376_i_at	J05428	UDP glycosyltransferase 2 family, polypeptide B7	0.00914	1.84647
36113_s_at	AJ011712	troponin T1, skeletal, slow	0.00241	1.83573
1591_s_at	J03242	insulin-like growth factor 2 (somatomedin A)	0.07240	1.82431
41783_at	M97815	cellular retinoic acid binding protein 2	0.00953	1.81372
37890_at	X69398	CD47 antigen (Rh-related antigen, integrin-associated signal transducer)	0.00080	1.81157
286_at	L19779	histone 2, H2aa	0.00313	1.81048
39677_at	X97671	KIAA0186 gene product	0.00245	1.79995
38116_at	D14657	KIAA0101 gene product	0.02134	1.79617
534_s_at	U20391	folate receptor 1 (adult)	0.00432	1.79504
39109_at	AB024704	TPX2, microtubule-associated protein homolog (Xenopus laevis)	0.01122	1.79492
37168_at	AB013924	lysosomal-associated membrane protein 3	0.01126	1.78649
2027_at	M87068	S100 calcium binding protein A2	0.00392	1.76465
38432_at	AA203213	interferon, alpha-inducible protein (clone IFI-15K)	0.00039	1.76152
37263_at	U55206	gamma-glutamyl hydrolase (conjugase, folylpolygammaglutamyl hydrolase)	0.00402	1.75929
35699_at	AB017430	BUB1 budding uninhibited by benzimidazoles 1 homolog beta (yeast)	0.00235	1.74772
926_at	J03910	metallothionein 1G	0.00037	1.74531
40412_at	AA203476	pituitary tumor-transforming 1	0.00009	1.74209
40541_at	X01630	argininosuccinate synthetase	0.00074	1.73492
649_s_at	L06797	chemokine (C-X-C motif) receptor 4	0.02078	1.72424
1603_g_at	L33881	protein kinase C, iota	0.01249	1.72282
34194_at	AL049313	NA	0.00198	1.72169
33436_at	Z46629	SRY (sex determining region Y)-box 9 (campomelic dysplasia, autosomal sex-reversal)	0.00004	1.72078
32787_at	M34309	v-erb-b2 erythroblastic leukemia viral oncogene homolog 3 (avian)	0.00381	1.71659
543_g_at	S74445	cellular retinoic acid binding protein 1	0.00155	1.69414
121_at	X68742	paired box gene 8	0.00091	1.68969
38763_at	L29254	sorbitol dehydrogenase	0.00689	1.67330
39660_at	X97671	defensin, beta 1	0.02558	1.67303
36643_at	L20817	discoidin domain receptor family, member 1	0.00101	1.66935
39728_at	J03909	interferon, gamma-inducible protein 30	0.01070	1.66164
33143_s_at	U81800	solute carrier family 16 (monocarboxylic acid transporters), member 3	0.00053	1.65206
39579_at	U89916	claudin 10	0.00955	1.65030
36879_at	M63193	endothelial cell growth factor 1 (platelet-derived)	0.00071	1.64496
1602_at	L33881	protein kinase C, iota	0.01202	1.64451
32609_at	AI885852	histone 2, H2aa	0.03471	1.64135
39175_at	D25328	phosphofructokinase, platelet	0.00239	1.63881
37985_at	L37747	lamin B1	0.00867	1.63785
40407_at	U28386	karyopherin alpha 2 (RAG cohort 1, importin alpha 1)	0.00314	1.63750
36813_at	U96131	thyroid hormone receptor interactor 13	0.00592	1.62873
1225_g_at	X66363	PCTAIRE protein kinase 1	0.01062	1.62485
32163_f_at	AA216639	NA	0.00696	1.62133

38545_at	M31682	inhibin, beta B (activin AB beta polypeptide)	0.07096	1.61934
2020_at	M73554	cyclin D1 (PRAD1	0.00877	1.60957
34743_at	D63481	scribble	0.00137	1.59423
36204_at	Y00815	protein tyrosine phosphatase, receptor type, F	0.00071	1.59381
38551_at	U52112	renin binding protein	0.01759	1.59193
41812_s_at	AB020713	nucleoporin 210	0.00012	1.59052
36101_s_at	M63978	vascular endothelial growth factor	0.00565	1.58999
33323_r_at	X57348	stratifin	0.00184	1.58898
925_at	J03909	interferon, gamma-inducible protein 30	0.00760	1.58895
38248_at	AB011124	ProSAPiP1 protein	0.01706	1.58870
33710_at	U72515	putative protein similar to nessy (Drosophila)	0.01118	1.58546
36108_at	M16276	major histocompatibility complex, class II, DQ beta 1	0.02411	1.57870
40746_at	L20814	glutamate receptor, ionotropic, AMPA 2	0.08211	1.56861
35756_at	AF089816	regulator of G-protein signalling 19 interacting protein 1	0.02303	1.56415
39610_at	X16665	homeo box B2	0.00032	1.56177
38978_at	AF013758	polyadenylate binding protein-interacting protein 1	0.03101	1.55990
36308_at	D76435	Zic family member 1 (odd-paired homolog, Drosophila)	0.14669	1.55561
32818_at	X78565	tenascin C (hexabrachion)	0.01302	1.55443
1620_at	D31784	cadherin 6, type 2, K-cadherin (fetal kidney)	0.05776	1.55222
39791_at	M23114	ATPase, Ca++ transporting, cardiac muscle, slow twitch 2	0.00307	1.54812
39043_at	AF006084	actin related protein 2/3 complex, subunit 1B, 41kDa	0.00390	1.54778
1224_at	X66363	PCTAIRE protein kinase 1	0.01408	1.54289
41355_at	N95229	B-cell CLL/lymphoma 11A (zinc finger protein)	0.00570	1.54115
2053_at	M34064	cadherin 2, type 1, N-cadherin (neuronal)	0.02566	1.54049
1945_at	M25753	cyclin B1	0.02435	1.53807
36446_s_at	L24521	hepatoma-derived growth factor (high-mobility group protein 1-like)	0.02083	1.53583
34390_at	U90441	procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha polypeptide II	0.00286	1.53152
41579_s_at	AI952267	protein kinase C, iota	0.00648	1.53010
40726_at	U37426	kinesin family member 11	0.00162	1.52805
408_at	X54131	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	0.00527	1.52736
37305_at	U61145	enhancer of zeste homolog 2 (Drosophila)	0.00035	1.52729
37117_at	Z83838	Rho GTPase activating protein 8	0.00063	1.52478
1521_at	X17620	non-metastatic cells 1, protein (NM23A) expressed in	0.00336	1.52346
38138_at	D38583	S100 calcium binding protein A11 (calgizzarin)	0.00205	1.52167
33661_at	U66589	ribosomal protein L5	0.01055	1.51688
41047_at	AI885170	chromosome 9 open reading frame 16	0.00221	1.51675
40347_at	AA913812	acidic (leucine-rich) nuclear phosphoprotein 32 family, member E	0.00065	1.51463
37782_at	AI636761	somatostatin	0.04020	1.51041
38546_at	AB006537	interleukin 1 receptor accessory protein	0.00797	1.50564
32880_at	AW015055	secretoglobin, family 1D, member 2	0.00304	1.50134
1083_s_at	M35093	mucin 1, transmembrane	0.00746	1.49359
35226_at	U71207	eyes absent homolog 2 (Drosophila)	0.02891	1.49310
860_at	U03911	mutS homolog 2, colon cancer, nonpolyposis type 1 (E. coli)	0.04414	1.48594
38853_at	X81892	G protein-coupled receptor 64	0.01116	1.48047
37749_at	D78611	mesoderm specific transcript homolog (mouse)	0.04367	1.47420
41400_at	K02581	thymidine kinase 1, soluble	0.00033	1.47195
41632_at	D38550	E2F transcription factor 3	0.00379	1.47048
37347_at	AA926959	CDC28 protein kinase regulatory subunit 1B	0.01438	1.46902
41356_at	W27619	B-cell CLL/lymphoma 11A (zinc finger protein)	0.10799	1.46895
38592_s_at	AI828210	NA	0.00263	1.46624
37302_at	U30872	centromere protein F, 350/400ka (mitosin)	0.00808	1.46584
37273_at	AF007153	NA	0.00521	1.46200
34512_at	J03853	adrenergic, alpha-2C-, receptor	0.00824	1.46059
36029_at	U57911	chromosome 11 open reading frame 8	0.01545	1.45539
37956_at	U37519	aldehyde dehydrogenase 3 family, member B2	0.02835	1.45532
39625_at	AL050204	NA	0.00898	1.45518
39829_at	AB016811	ADP-ribosylation factor-like 7	0.00235	1.45385
35829_at	AL080181	immunoglobulin superfamily, member 4	0.04575	1.44866

41310_f_at	X12794	nuclear receptor subfamily 2, group F, member 6	0.00027	1.44827
425_at	X67325	interferon, alpha-inducible protein 27	0.04049	1.44215
37310_at	X02419	plasminogen activator, urokinase	0.03443	1.43447
32134_at	AL050162	testis derived transcript (3 LIM domains)	0.00849	1.43182
35769_at	AJ011001	G protein-coupled receptor 56	0.00002	1.42600
39073_at	AL038662	non-metastatic cells 1, protein (NM23A) expressed in	0.00336	1.41893
35277_at	AB018305	spondin 1, (f-spondin) extracellular matrix protein	0.01476	1.41656
35778_at	AB011103	kinesin family member 5C	0.02658	1.41170
37343_at	U01062	inositol 1,4,5-triphosphate receptor, type 3	0.00021	1.40987
1914_at	U66838	cyclin A1	0.01191	1.40931
36651_at	X15525	acid phosphatase 2, lysosomal	0.00452	1.40803
1368_at	M27492	interleukin 1 receptor, type I	0.00632	1.40696
38086_at	AB007935	immunoglobulin superfamily, member 3	0.00691	1.40183
36821_at	AL050367	hypothetical protein LOC221061	0.10519	1.38749
40445_at	AF017307	E74-like factor 3 (ets domain transcription factor, epithelial-specific)	0.01387	1.38084
904_s_at	L47276	topoisomerase (DNA) II alpha 170kDa	0.00107	1.37950
37200_at	J04162	Fc fragment of IgG, low affinity IIIa, receptor for (CD16)	0.05250	1.37816
182_at	S82470	inositol 1,4,5-triphosphate receptor, type 3	0.00035	1.37682
1218_at	X12794	nuclear receptor subfamily 2, group F, member 6	0.00534	1.37250
36658_at	D13643	24-dehydrocholesterol reductase	0.02935	1.36964
40134_at	AF047436	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit f, isoform 2	0.02582	1.36188
38143_at	L33404	kallikrein 7 (chymotryptic, stratum corneum)	0.03574	1.36161
37218_at	D64110	BTG family, member 3	0.00423	1.35371
36497_at	W28438	NA	0.00370	1.35310
1985_s_at	X73066	non-metastatic cells 1, protein (NM23A) expressed in	0.00393	1.35050
36761_at	AL079276	zinc finger protein 339	0.00004	1.34994
36782_s_at	J03242	insulin-like growth factor 2 (somatomedin A)	0.13410	1.34344
36070_at	AL049389	KIAA1199 protein	0.00083	1.33864
32140_at	Y08110	sortilin-related receptor, L(DLR class) A repeats-containing	0.00830	1.33334
613_at	M21389	keratin 5 (epidermolysis bullosa simplex, Dowling-Meara/Kobner/Weber-Cockayne types)	0.00888	1.33264
40899_at	Y00503	keratin 19	0.04301	1.32738
34348_at	U78095	serine protease inhibitor, Kunitz type, 2	0.00601	1.32600
32317_s_at	U34804	sulfotransferase family, cytosolic, 1A, phenol-preferring, member 2	0.00765	1.32555
36484_at	AI935146	UDP-N-acetyl-alpha-D-galactosamine	0.00112	1.32439
32137_at	AF029778	jagged 2	0.01487	1.32362
895_at	L19686	macrophage migration inhibitory factor (glycosylation-inhibiting factor)	0.00513	1.31965
1402_at	M16038	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog	0.00644	1.31894
1490_at	M19720	v-myc myelocytomatosis viral oncogene homolog 1, lung carcinoma derived (avian)	0.00497	1.31885
38391_at	M94345	capping protein (actin filament), gelsolin-like	0.01717	1.31658
36457_at	U10860	guanine monphosphate synthetase	0.00496	1.31602
40145_at	AI375913	topoisomerase (DNA) II alpha 170kDa	0.00516	1.31519
37883_i_at	AI375033	hypothetical protein AF038169	0.02814	1.31352
32559_s_at	AJ238096	LSM4 homolog, U6 small nuclear RNA associated (S. cerevisiae)	0.00637	1.31015
33920_at	AF051782	diaphanous homolog 1 (Drosophila)	0.00013	1.30575
38158_at	D79987	extra spindle poles like 1 (S. cerevisiae)	0.00005	1.30475
1877_g_at	M29893	v-ral simian leukemia viral oncogene homolog A (ras related)	0.04273	1.30296
35803_at	S82240	ras homolog gene family, member E	0.04300	1.30268
38411_at	U90916	sortilin-related receptor, L(DLR class) A repeats-containing	0.04444	1.29505
36610_at	D21852	R3H domain (binds single-stranded nucleic acids) containing	0.01735	1.29282
32263_at	AL080146	cyclin B2	0.00268	1.29103
40041_at	AF017790	highly expressed in cancer, rich in leucine heptad repeats	0.00932	1.29044
1713_s_at	U26727	cyclin-dependent kinase inhibitor 2A (melanoma, p16, inhibits CDK4)	0.04628	1.28546

628_at	L37882	frizzled homolog 2 (Drosophila)	0.01558	1.28295
37360_at	U66711	lymphocyte antigen 6 complex, locus E	0.01072	1.27492
33130_at	AW001001	homeo box D1	0.03137	1.27400
37961_at	U90907	phosphoinositide-3-kinase, regulatory subunit, polypeptide 3 (p55, gamma)	0.03890	1.27165
34563_at	D26361	kinesin family member 14	0.00393	1.26557
38111_at	X15998	chondroitin sulfate proteoglycan 2 (versican)	0.16088	1.26490
741_g_at	D49394	5-hydroxytryptamine (serotonin) receptor 3A	0.08229	1.26452
34852_g_at	AF011468	serine/threonine kinase 6	0.00066	1.25970
41688_at	AI688299	transmembrane 4 superfamily member 11 (plasmolipin)	0.00420	1.25919
32767_at	M74558	TAL1 (SCL) interrupting locus	0.01290	1.25897
32051_at	AJ224875	asparagine-linked glycosylation 8 homolog (yeast, alpha-1,3-glucosyltransferase)	0.01614	1.25778
431_at	X02530	chemokine (C-X-C motif) ligand 10	0.10188	1.25688
36167_at	D89052	ATPase, H+ transporting, lysosomal 21kDa, V0 subunit c"	0.00172	1.25633
1385_at	M77349	transforming growth factor, beta-induced, 68kDa	0.08975	1.25396
41585_at	AB018289	KIAA0746 protein	0.00504	1.25372
36454_at	AF037335	carbonic anhydrase XII	0.05908	1.25176
32156_at	AF044968	poliovirus receptor-related 2 (herpesvirus entry mediator B)	0.00091	1.24900
37784_at	AL049227	NA	0.00934	1.24887
34308_at	U90551	histone 1, H2ac	0.02767	1.24540
34736_at	M25753	cyclin B1	0.01274	1.24260
32959_at	M25809	ATPase, H+ transporting, lysosomal 56/58kDa, V1 subunit B, isoform 1 (Renal tubular acidosis with deafness)	0.01109	1.24078
41732_at	AA310786	similar to My016 protein	0.00074	1.23986
35807_at	M21186	cytochrome b-245, alpha polypeptide	0.00042	1.23976
36927_at	AB000115	chromosome 1 open reading frame 29	0.08658	1.23739
38313_at	AB028985	ATP-binding cassette, sub-family A (ABC1), member 2	0.00164	1.23620
33272_at	AA829286	serum amyloid A1	0.04305	1.22919
35631_at	U37689	polymerase (RNA) II (DNA directed) polypeptide H	0.00320	1.22596
39396_at	AF081281	lysophospholipase I	0.07865	1.22335
41168_at	AF029750	TAP binding protein (tapasin)	0.01571	1.21901
39092_at	AW007731	histone H2A.F/Z variant	0.04113	1.21844
34693_at	D13814	sialyltransferase	0.00117	1.21690
41060_at	M74093	cyclin E1	0.01429	1.21674
36499_at	Z16411	cadherin, EGF LAG seven-pass G-type receptor 2 (flamingo homolog, Drosophila)	0.00099	1.21557
41470_at	AF027208	prominin 1	0.02018	1.21483
34177_at	AF038660	UDP-Gal	0.00813	1.20943
1007_s_at	U48705	discoidin domain receptor family, member 1	0.00387	1.20073
33484_at	Y10571	ring finger protein 2	0.03278	1.19997
35828_at	D42123	cysteine-rich protein 2	0.01135	1.19894
38087_s_at	W72186	S100 calcium binding protein A4 (calcium protein, calvasculin, metastasin, murine placental homolog)	0.03813	1.19807
38442_at	U19718	microfibrillar-associated protein 2	0.10931	1.19697
39056_at	X53793	phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoimidazole succinocarboxamide synthetase	0.01290	1.19551
32509_at	AI307607	HBxAg transactivated protein 2	0.04726	1.19368
38576_at	AJ223353	histone 1, H2bd	0.03352	1.19352
40303_at	U85658	transcription factor AP-2 gamma (activating enhancer binding protein 2 gamma)	0.00872	1.19238
39219_at	U20240	CCAAT/enhancer binding protein (C/EBP), gamma	0.03060	1.19115
38123_at	D14878	chromosome 10 open reading frame 7	0.03775	1.18766
34251_at	M92299	homeo box B5	0.03894	1.18722
34970_r_at	AI655458	5-oxoprolinase (ATP-hydrolysing)	0.00253	1.18706
39253_s_at	M29893	v-ral simian leukemia viral oncogene homolog A (ras related)	0.03881	1.18588
33253_at	D50919	tripartite motif-containing 14	0.03258	1.18490
32963_s_at	W27549	Ras-related GTP binding D	0.03099	1.18400
36512_at	L32179	arylacetamide deacetylase (esterase)	0.00847	1.18214
34715_at	U74612	forkhead box M1	0.00102	1.17668
41610_at	AB011105	laminin, alpha 5	0.02048	1.17590

38161_at	Y09022	asparagine-linked glycosylation 3 homolog (yeast, alpha-1,3-mannosyltransferase)	0.00188	1.17313
41696_at	AI620381	chromosome 7 open reading frame 24	0.00532	1.17054
40888_f_at	W28170	eukaryotic translation elongation factor 1 alpha 1	0.07186	1.16916
40193_at	X68985	enolase 2, (gamma, neuronal)	0.01002	1.16887
41188_at	W28186	lysosomal associated protein transmembrane 4 beta	0.01470	1.16852
37727_i_at	X78669	reticulocalbin 2, EF-hand calcium binding domain	0.04904	1.16792
35858_at	AA996066	PRKR interacting protein 1 (IL11 inducible)	0.01383	1.16698
41193_at	AB013382	dual specificity phosphatase 6	0.00873	1.16528
37761_at	AB015020	BAI1-associated protein 2	0.00317	1.16385
34699_at	D13814	CD2-associated protein	0.04690	1.16155
36898_r_at	X74331	primase, polypeptide 2A, 58kDa	0.02863	1.15825
38160_at	AF011333	lymphocyte antigen 75	0.02219	1.15686
41189_at	Y09392	tumor necrosis factor receptor superfamily, member 25	0.00253	1.15560
39183_at	X66363	PCTAIRE protein kinase 1	0.01779	1.15519
1173_g_at	M77693	spermidine/spermine N1-acetyltransferase	0.01001	1.15094
40788_at	U84371	adenylate kinase 2	0.00129	1.15029
38336_at	AB023230	GRP1-binding protein GRSP1	0.03611	1.15024
39353_at	AI912041	heat shock 10kDa protein 1 (chaperonin 10)	0.05187	1.15018
41058_g_at	AI760162	thioesterase superfamily member 2	0.03412	1.14864
36145_at	U51586	fuse-binding protein-interacting repressor	0.01590	1.14795
2035_s_at	M55914	enolase 1, (alpha)	0.00102	1.14710
36804_at	M34455	indoleamine-pyrrole 2,3 dioxygenase	0.08612	1.14543
36575_at	S59049	regulator of G-protein signalling 1	0.06883	1.14305
35615_at	D50914	block of proliferation 1	0.00352	1.14255
33702_f_at	L05144	phosphoenolpyruvate carboxykinase 1 (soluble)	0.00310	1.14237
1803_at	X05360	cell division cycle 2, G1 to S and G2 to M	0.01169	1.14114
31935_s_at	U75968	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 11 (CHL1-like helicase homolog, S. cerevisiae)	0.00018	1.14095
38618_at	AC002073	HGFL gene	0.00408	1.14035
36581_at	U09510	glycyl-tRNA synthetase	0.02578	1.13487
35320_at	AB004857	solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2	0.01757	1.13329
31600_s_at	D38435	postmeiotic segregation increased 2-like 1	0.05851	1.13270
41096_at	AI126134	S100 calcium binding protein A8 (calgranulin A)	0.03939	1.13021
34661_at	AB002348	KIAA0350 protein	0.00916	1.12959
36561_at	X73424	propionyl Coenzyme A carboxylase, beta polypeptide	0.02912	1.12685
41198_at	AF055008	granulin	0.00137	1.12658
1451_s_at	U80987	osteoblast specific factor 2 (fasciclin I-like)	0.21497	1.12399
41485_at	X02152	lactate dehydrogenase A	0.00695	1.12267
38257_at	AF038406	NADH dehydrogenase (ubiquinone) Fe-S protein 8, 23kDa (NADH-coenzyme Q reductase)	0.00686	1.12082
821_s_at	U78793	folate receptor 1 (adult)	0.11578	1.12047
40434_at	U97519	podocalyxin-like	0.00541	1.12015
41223_at	M22760	cytochrome c oxidase subunit Va	0.03355	1.12013
37677_at	V00572	phosphoglycerate kinase 1	0.02627	1.11657
35630_at	X87342	lethal giant larvae homolog 2 (Drosophila)	0.00006	1.11489
34304_s_at	AL050290	spermidine/spermine N1-acetyltransferase	0.01292	1.11480
34865_at	AI360249	NADH dehydrogenase (ubiquinone) Fe-S protein 6, 13kDa (NADH-coenzyme Q reductase)	0.03250	1.11476
36863_at	AF032862	hyaluronan-mediated motility receptor (RHAMM)	0.00196	1.11396
33247_at	U86782	proteasome (prosome, macropain) 26S subunit, non-ATPase, 14	0.04130	1.10643
37603_at	X52015	interleukin 1 receptor antagonist	0.00991	1.10624
39812_at	X79865	mitochondrial ribosomal protein L12	0.00132	1.10555
41451_s_at	W28498	SAR1a gene homolog 1 (S. cerevisiae)	0.09838	1.10390
39311_at	AF000381	folate receptor 1 (adult)	0.03019	1.10359
40417_at	D43950	chaperonin containing TCP1, subunit 5 (epsilon)	0.03949	1.10101
33266_at	AF015254	aurora kinase B	0.00035	1.10065
38967_at	AF054175	chromosome 14 open reading frame 2	0.08320	1.10032
40536_f_at	AI254524	translation initiation factor IF2	0.00431	1.10031
41583_at	AC004770	flap structure-specific endonuclease 1	0.03718	1.09851
295_s_at	U14755	LIM homeobox 1	0.01326	1.09326

1599_at	L25876	cyclin-dependent kinase inhibitor 3 (CDK2-associated dual specificity phosphatase)	0.01271	1.09248
34885_at	AJ002308	synaptogyrin 2	0.00194	1.09095
32034_at	AF041259	zinc finger protein 217	0.05755	1.08850
33374_at	L09708	complement component 2	0.06814	1.08817
41084_at	AI659108	hypothetical protein MGC51082	0.07846	1.08784
34878_at	AB019987	SMC4 structural maintenance of chromosomes 4-like 1 (yeast)	0.02926	1.08578
40161_at	L32137	cartilage oligomeric matrix protein (pseudoachondroplasia, epiphyseal dysplasia 1, multiple)	0.05491	1.08559
38429_at	U29344	fatty acid synthase	0.00428	1.08525
33132_at	U37012	cleavage and polyadenylation specific factor 1, 160kDa	0.00014	1.08488
38949_at	L01087	protein kinase C, theta	0.00540	1.08431
38389_at	X04371	2',5'-oligoadenylate synthetase 1, 40/46kDa	0.06910	1.08408
39158_at	AB021663	activating transcription factor 5	0.00143	1.08293
32579_at	U29175	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4	0.03963	1.07996
33260_at	L13857	son of sevenless homolog 1 (Drosophila)	0.00264	1.07962
37926_at	D14520	Kruppel-like factor 5 (intestinal)	0.00254	1.07940
35227_at	U72066	retinoblastoma binding protein 8	0.02136	1.07924
38647_at	AJ131182	coatomer protein complex, subunit epsilon	0.00749	1.07769
39969_at	AA255502	histone 1, H4c	0.11568	1.07559
1242_at	U15655	Ets2 repressor factor	0.00363	1.07458
35844_at	D79206	syndecan 4 (amphiglycan, ryudocan)	0.00387	1.07354
38112_g_at	X15998	chondroitin sulfate proteoglycan 2 (versican)	0.18393	1.07280
36811_at	U24389	lysyl oxidase-like 1	0.07861	1.07229
32332_at	X69433	isocitrate dehydrogenase 2 (NADP+), mitochondrial	0.00162	1.07155
37984_s_at	M57763	ADP-ribosylation factor 6	0.15424	1.07121
39105_at	Z46389	vasodilator-stimulated phosphoprotein	0.00826	1.07048
40535_i_at	AI254524	translation initiation factor IF2	0.01108	1.07019
37686_s_at	Y09008	uracil-DNA glycosylase	0.03756	1.06374
33212_at	AF006751	ribosome binding protein 1 homolog 180kDa (dog)	0.01116	1.06343
1979_s_at	U29656	nucleolar protein 1, 120kDa	0.00943	1.06326
36008_at	AF041434	protein tyrosine phosphatase type IVA, member 3	0.00209	1.06321
35666_at	U38276	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3F	0.01827	1.06289
757_at	D28364	annexin A2	0.00944	1.05996
35703_at	X06374	platelet-derived growth factor alpha polypeptide	0.03963	1.05929
32154_at	M36711	transcription factor AP-2 alpha (activating enhancer binding protein 2 alpha)	0.03236	1.05915
34795_at	U84573	procollagen-lysine, 2-oxoglutarate 5-dioxygenase (lysine hydroxylase) 2	0.11881	1.05760
1592_at	J04088	topoisomerase (DNA) II alpha 170kDa	0.01884	1.05644
2088_s_at	M94151	EphB2	0.05732	1.05558
41320_s_at	U69609	leucine rich repeat (in FLII) interacting protein 1	0.04510	1.05496
32796_f_at	U66061	trypsinogen C	0.03956	1.05416
36957_at	W22296	protein kinase C binding protein 1	0.02970	1.05373
41250_at	U24169	JTV1 gene	0.00254	1.05260
37740_r_at	J02683	solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 5	0.04286	1.05169
36687_at	N50520	cytochrome c oxidase subunit VIIb	0.07891	1.05014
35576_f_at	AL009179	putative G-protein coupled receptor pseudogene	0.05843	1.04933
41045_at	U77643	secreted and transmembrane 1	0.06572	1.04919
32622_at	L36983	dynamin 2	0.00004	1.04644
37576_at	U52969	Purkinje cell protein 4	0.05114	1.04637
36838_at	AF055481	kallikrein 10	0.03228	1.04608
36135_at	U86602	EBNA1 binding protein 2	0.00944	1.04565
36598_s_at	L36818	inositol polyphosphate phosphatase-like 1	0.00105	1.04434
40504_at	AF001601	paraoxonase 2	0.02857	1.04417
1741_s_at	S37730	insulin-like growth factor binding protein 2, 36kDa	0.04851	1.04312
32670_at	L38969	thrombospondin 3	0.00542	1.04151
38617_at	D45906	LIM domain kinase 2	0.00097	1.04128
38127_at	Z48199	syndecan 1	0.01473	1.04095

1616_at	U44105	fibroblast growth factor 9 (glia-activating factor)	0.14962	1.04072
40195_at	X68985	H2A histone family, member X	0.08174	1.04068
36155_at	D87465	sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican) 2	0.00180	1.04039
34022_at	M36821	chemokine (C-X-C motif) ligand 3	0.00018	1.03837
36388_at	U25128	parathyroid hormone receptor 2	0.03885	1.03825
40813_at	AI768188	mitochondrial ribosomal protein S6	0.09149	1.03693
39817_s_at	AF040105	putative c-Myc-responsive	0.00862	1.03675
32859_at	M97935	signal transducer and activator of transcription 1, 91kDa	0.02852	1.03619
40422_at	X16302	insulin-like growth factor binding protein 2, 36kDa	0.09028	1.03543
41733_at	AC003007	KIAA0220 protein	0.07920	1.03468
38264_at	U74324	RAB interacting factor	0.00789	1.03432
36591_at	X06956	tubulin, alpha 1 (testis specific)	0.00991	1.03416
33791_at	Y15227	deleted in lymphocytic leukemia, 1	0.02683	1.03280
31859_at	J05070	matrix metalloproteinase 9 (gelatinase B, 92kDa gelatinase, 92kDa type IV collagenase)	0.01469	1.02883
36885_at	L28824	spleen tyrosine kinase	0.00098	1.02824
39837_s_at	AC004877	KIAA0543 protein	0.00108	1.02606
330_s_at	Z14227	tubulin, alpha 1 (testis specific)	0.00867	1.02324
37162_at	S72869	DNA segment on chromosome 10 (unique) 170	0.14687	1.02112
32046_at	D10495	protein kinase C, delta	0.02677	1.02102
32616_at	M16038	v-yes-1 Yamaguchi sarcoma viral related oncogene homolog	0.01885	1.01941
41073_at	AI743745	G protein-coupled receptor 49	0.07575	1.01816
34301_r_at	Z19574	keratin 17	0.00100	1.01725
40167_s_at	AF038187	WD repeat and SOCS box containing protein 2	0.11648	1.01659
34472_at	AB012911	frizzled homolog 6 (Drosophila)	0.03319	1.01599
38328_at	H10201	solute carrier family 25, member 13 (citrin)	0.01348	1.01507
32378_at	M26252	pyruvate kinase, muscle	0.00446	1.01442
2047_s_at	M74297	junction plakoglobin	0.02540	1.01410
40763_at	U85707	Meis1, myeloid ecotropic viral integration site 1 homolog (mouse)	0.01658	1.01288
38661_at	X75315	RNA-binding region (RNP1, RRM) containing 1	0.00183	1.01239
2054_g_at	M34064	cadherin 2, type 1, N-cadherin (neuronal)	0.06239	1.00964
41171_at	D45248	proteasome (prosome, macropain) activator subunit 2 (PA28 beta)	0.02266	1.00953
39342_at	X94754	methionine-tRNA synthetase	0.00205	1.00834
36676_at	AL031659	chromosome 20 open reading frame 132	0.08707	1.00728
32801_at	AB002315	KIAA0317 gene product	0.00192	1.00539
31523_f_at	Z80780	histone 1, H2be	0.02069	1.00193
1630_s_at	Z29630	spleen tyrosine kinase	0.00246	1.00188

Table 3.2. Genes over-expressed in omental metastases compared to primary ovarian serous adenocarcinomas.

Average difference and p values are shown.

Probe set	Acc no.	Gene description	P-value	Avg. diff
38430_at	AA128249	fatty acid binding protein 4, adipocyte	0.83304	5.18163
40657_r_at	H15814	adipose most abundant gene transcript 1	0.13398	3.74608
37122_at	AB005293	perilipin	0.53899	3.48806
41209_at	M15856	lipoprotein lipase	0.04191	2.83884
33273_f_at	X57809	immunoglobulin lambda locus	0.34906	2.69798
35730_at	X03350	alcohol dehydrogenase IB (class I), beta polypeptide	0.72553	2.67887
33274_f_at	M18645	immunoglobulin lambda joining 3	0.45705	2.63531
38326_at	M69199	putative lymphocyte G0/G1 switch gene	0.53372	2.42394
37864_s_at	Y14737	immunoglobulin heavy constant gamma 3 (G3m marker)	0.90520	2.26880
33272_at	AA829286	serum amyloid A1	0.68327	2.15547
38299_at	X04430	interleukin 6 (interferon, beta 2)	0.39328	2.09411
37032_at	U08021	nicotinamide N-methyltransferase	0.83388	2.05498
37565_at	X85750	monocyte to macrophage differentiation-associated	0.19630	1.99608
38038_at	U21128	lumican	0.23649	1.99192
36984_f_at	X89214	haptoglobin-related protein	0.87366	1.96664
41827_f_at	AI932613	hypothetical protein LOC51233	0.82525	1.96001
40082_at	D10040	fatty-acid-Coenzyme A ligase, long-chain 2	0.55850	1.92182
41164_at	X67301	immunoglobulin heavy constant mu	0.57179	1.83487
32243_g_at	AL038340	crystallin, alpha B	0.84334	1.82972
35334_at	U94362	glycogenin 2	0.64430	1.81367
32805_at	U05861	aldo-keto reductase family 1, member C1 (dihydrodiol dehydrogenase 1; 20-alpha (3-alpha)-hydroxysteroid dehydrogenase)	0.89452	1.81327
40282_s_at	M84526	D component of complement (adipsin)	0.18182	1.78729
37006_at	AI660656	immunoglobulin J polypeptide, linker protein for immunoglobulin alpha and mu polypeptides	0.74250	1.77665
32552_at	X00129	retinol binding protein 4, plasma	0.35500	1.76447
34637_f_at	M12963	alcohol dehydrogenase 1A (class I), alpha polypeptide	0.06184	1.75604
41772_at	M68840	monoamine oxidase A	0.33343	1.68798
819_at	U76456	tissue inhibitor of metalloproteinase 4	0.59229	1.66460
32242_at	AL038340	crystallin, alpha B	0.00919	1.65875
36686_at	U07919	aldehyde dehydrogenase 1 family, member A3	0.97414	1.65696
32542_at	AF063002	four and a half LIM domains 1	0.05803	1.65223
32527_at	AI381790	adipose specific 2	0.24944	1.64180
37221_at	M31158	protein kinase, cAMP-dependent, regulatory, type II, beta	0.69461	1.63584
41771_g_at	AA420624	monoamine oxidase A	0.25128	1.63101
37954_at	X16662	annexin A8	0.81388	1.62047
38194_s_at	M63438	NA	0.19657	1.62039
36119_at	AF070648	caveolin 1, caveolae protein, 22kDa	0.59093	1.58713
40658_r_at	D45371	adipose most abundant gene transcript 1	0.75835	1.55862
190_at	U09937	nuclear receptor subfamily 4, group A, member 3	0.62999	1.44802
160030_at	X06562	growth hormone receptor	0.34167	1.41245
39031_at	AA152406	cytochrome c oxidase subunit VIIa polypeptide 1 (muscle)	0.76618	1.40486
1814_at	D50683	transforming growth factor, beta receptor II (70/80kDa)	0.70846	1.40278
39673_i_at	X97671	extracellular matrix protein 2, female organ and adipocyte specific	0.64976	1.38750
32488_at	X14420	collagen, type III, alpha 1 (Ehlers-Danlos syndrome type IV, autosomal dominant)	0.77319	1.37023
37671_at	S78569	laminin, alpha 4	0.38676	1.36187
36606_at	X51405	carboxypeptidase E	0.46794	1.35424
296_at	X79535	tubulin, beta polypeptide	0.39319	1.34455
35926_s_at	AF004230	leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 1	0.36236	1.34442
32747_at	X05409	aldehyde dehydrogenase 2 family (mitochondrial)	0.14789	1.33655
37157_at	X56667	calbindin 2, 29kDa (calretinin)	0.21031	1.33111
40841_at	AF049910	transforming, acidic coiled-coil containing protein 1	0.22341	1.32999
41385_at	AB023204	erythrocyte membrane protein band 4.1-like 3	0.21258	1.32431
36983_f_at	X00442	haptoglobin	0.61263	1.31547

31687_f_at	M25079	hemoglobin, beta	0.24644	1.31382
38379_at	X76534	glycoprotein (transmembrane) nmb	0.48483	1.28754
39114_at	AB022718	decidual protein induced by progesterone	0.79641	1.27741
32664_at	D37931	ribonuclease, RNase A family, 4	0.83442	1.27635
37215_at	AF046798	phosphorylase, glycogen; liver (Hers disease, glycogen storage disease type VI)	0.79541	1.27147
37951_at	AF035119	deleted in liver cancer 1	0.60724	1.26986
37279_at	U10550	GTP binding protein overexpressed in skeletal muscle	0.34465	1.25872
339_at	AF005887	caveolin 2	0.52424	1.25705
40659_at	U12767	nuclear receptor subfamily 4, group A, member 3	0.43938	1.25444
32227_at	X17042	proteoglycan 1, secretory granule	0.27930	1.24057
38052_at	M14539	coagulation factor XIII, A1 polypeptide	0.96497	1.22270
1815_g_at	D50683	transforming growth factor, beta receptor II (70/80kDa)	0.36818	1.20849
33702_f_at	L05144	phosphoenolpyruvate carboxykinase 1 (soluble)	0.78227	1.20112
32551_at	U03877	EGF-containing fibulin-like extracellular matrix protein 1	0.87925	1.20028
36627_at	X86693	SPARC-like 1 (mast9, hevin)	0.08451	1.19862
38717_at	AL050159	DKFZP586A0522 protein	0.17110	1.19037
658_at	L12350	thrombospondin 2	0.63158	1.18974
34793_s_at	M22299	plastin 3 (T isoform)	0.09126	1.18566
40071_at	U03688	cytochrome P450, family 1, subfamily B, polypeptide 1	0.89737	1.18504
38786_at	AL079279	likely ortholog of mouse polydom	0.51225	1.18355
35704_at	X92814	HRAS-like suppressor 3	0.21244	1.17772
39350_at	U50410	glypican 3	0.47079	1.17404
1451_s_at	U80987	osteoblast specific factor 2 (fasciclin I-like)	0.36087	1.17125
479_at	U53446	disabled homolog 2, mitogen-responsive phosphoprotein (Drosophila)	0.50660	1.17051
37630_at	AL049176	neuralin 1	0.75663	1.16027
40077_at	Z11559	aconitase 1, soluble	0.34717	1.15561
32052_at	L48215	hemoglobin, beta	0.37760	1.15239
38378_at	M37033	CD53 antigen	0.05886	1.15072
1403_s_at	M21121	chemokine (C-C motif) ligand 5	0.19086	1.14807
38466_at	X82153	cathepsin K (pycnodysostosis)	0.09103	1.13912
32538_at	S95936	transferrin	0.48220	1.13290
36681_at	J02611	apolipoprotein D	0.73660	1.12565
39616_at	AL050227	prostaglandin E receptor 3 (subtype EP3)	0.18890	1.12307
35303_at	U96876	insulin induced gene 1	0.68307	1.12170
39333_at	M26576	collagen, type IV, alpha 1	0.24210	1.12124
35785_at	W28281	GABA(A) receptor-associated protein like 1	0.06910	1.10516
40496_at	J04080	complement component 1, s subcomponent	0.44320	1.10184
859_at	U03688	cytochrome P450, family 1, subfamily B, polypeptide 1	0.46561	1.09714
37015_at	K03000	aldehyde dehydrogenase 1 family, member A1	0.54460	1.09559
33902_at	L34041	glycerol-3-phosphate dehydrogenase 1 (soluble)	0.86058	1.09193
34311_at	X76648	glutaredoxin (thioltransferase)	0.23254	1.08808
37104_at	L40904	peroxisome proliferative activated receptor, gamma	0.94652	1.08614
34797_at	AF014402	phosphatidic acid phosphatase type 2A	0.22838	1.08183
41165_g_at	X67301	immunoglobulin heavy constant mu	0.17729	1.08008
40375_at	X63741	early growth response 3	0.55743	1.07870
32755_at	X13839	actin, alpha 2, smooth muscle, aorta	0.78592	1.06898
37398_at	AA100961	platelet/endothelial cell adhesion molecule (CD31 antigen)	0.07307	1.06559
41123_s_at	L35594	ectonucleotide pyrophosphatase/phosphodiesterase 2 (autotaxin)	0.18518	1.05975
40202_at	D31716	basic transcription element binding protein 1	0.74951	1.05402
37908_at	U31384	guanine nucleotide binding protein (G protein), gamma 11	0.13786	1.05386
40856_at	U29953	serine (or cysteine) proteinase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1	0.93324	1.05227
41096_at	AI126134	S100 calcium binding protein A8 (calgranulin A)	0.54709	1.05122
37762_at	Y07909	epithelial membrane protein 1	0.84863	1.04912
33890_at	AF005887	regulator of G-protein signalling 5	0.74310	1.04778
34303_at	AL049949	hypothetical protein FLJ90798	0.36417	1.04672
408_at	X54131	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	0.49865	1.04631
38220_at	U20938	dihydropyrimidine dehydrogenase	0.41881	1.04527
36543_at	J02931	coagulation factor III (thromboplastin, tissue factor)	0.38411	1.04507

659_g_at	L12350	thrombospondin 2	0.49672	1.04362
37187_at	M36820	chemokine (C-X-C motif) ligand 2	0.91301	1.03605
38653_at	D11428	peripheral myelin protein 22	0.05092	1.03595
41504_s_at	AF055376	v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)	0.47673	1.03343
32612_at	X04412	gelsolin (amyloidosis, Finnish type)	0.19343	1.03305
40419_at	X85116	stomatin	0.78835	1.03231
40518_at	Y00062	protein tyrosine phosphatase, receptor type, C	0.35216	1.03203
41770_at	AA420624	monoamine oxidase A	0.13208	1.03064
34375_at	M28225	chemokine (C-C motif) ligand 2	0.44729	1.02818
36227_at	AF043129	interleukin 7 receptor	0.46876	1.02437
844_at	U48707	protein phosphatase 1, regulatory (inhibitor) subunit 1A	0.82696	1.02085
39945_at	U09278	fibroblast activation protein, alpha	0.57437	1.01594
40456_at	AL049963	solute carrier family 39 (metal ion transporter), member 8	0.84436	1.00622
37185_at	Y00630	serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 2	0.81569	1.00611
39331_at	X79535	tubulin, beta polypeptide	0.97246	1.00473
37407_s_at	AF013570	myosin, heavy polypeptide 11, smooth muscle	0.36565	1.00273
37842_at	AF054589	I-mfa domain-containing protein	0.34788	1.00052
1105_s_at	M12886	T cell receptor beta locus	0.57650	0.99848
37701_at	L13463	regulator of G-protein signalling 2, 24kDa	0.04506	0.99524
40398_s_at	AI743406	mesenchyme homeo box 2 (growth arrest-specific homeo box)	0.51023	0.99475
32666_at	U19495	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	0.03370	0.99384
38427_at	L25286	collagen, type XV, alpha 1	0.52617	0.99230
38096_f_at	M83664	major histocompatibility complex, class II, DP beta 1	0.52742	0.99195
37599_at	Z84718	aldehyde oxidase 1	0.79059	0.98266
33756_at	U39447	amine oxidase, copper containing 3 (vascular adhesion protein 1)	0.41119	0.97798
38363_at	W60864	TYRO protein tyrosine kinase binding protein	0.12534	0.97567
1717_s_at	U45878	baculoviral IAP repeat-containing 3	0.78374	0.97352
614_at	M22430	phospholipase A2, group IIA (platelets, synovial fluid)	0.30086	0.96972
1372_at	M31165	tumor necrosis factor, alpha-induced protein 6	0.05372	0.96971
39568_g_at	AB006190	aquaporin 7	0.09472	0.96835
AFFX-BioDn-5_at	J04423	NA	0.26944	0.96745
32535_at	X63556	fibrillin 1 (Marfan syndrome)	0.17916	0.96562
33703_f_at	L12760	phosphoenolpyruvate carboxykinase 1 (soluble)	0.22253	0.96491
36617_at	X77956	inhibitor of DNA binding 1, dominant negative helix-loop-helix protein	0.24602	0.96441
38095_i_at	M83664	major histocompatibility complex, class II, DP beta 1	0.81686	0.95950
37402_at	D26129	ribonuclease, RNase A family, 1 (pancreatic)	0.54967	0.95694
875_g_at	M26683	chemokine (C-C motif) ligand 2	0.55219	0.95621
37724_at	V00568	v-myc myelocytomatosis viral oncogene homolog (avian)	0.12227	0.95473
1370_at	M29696	interleukin 7 receptor	0.38951	0.94853
996_at	X59065	fibroblast growth factor 1 (acidic)	0.82072	0.94779
32855_at	L00352	low density lipoprotein receptor (familial hypercholesterolemia)	0.81001	0.94606
36247_f_at	M12272	alcohol dehydrogenase 1C (class I), gamma polypeptide	0.42675	0.94441
1788_s_at	U48807	dual specificity phosphatase 4	0.95242	0.94343
38059_g_at	Z22865	dermatopontin	0.71328	0.94064
36073_at	U35139	necdin homolog (mouse)	0.94366	0.93991
39597_at	AB020650	KIAA0843 protein	0.30672	0.93553
32686_at	D86096	prostaglandin E receptor 3 (subtype EP3)	0.67392	0.93520
38111_at	X15998	chondroitin sulfate proteoglycan 2 (versican)	0.68240	0.93174
33821_at	AL034374	ELOVL family member 5, elongation of long chain fatty acids (FEN1/Elo2, SUR4/Elo3-like, yeast)	0.70269	0.93159
37203_at	L07765	carboxylesterase 1 (monocyte/macrophage serine esterase 1)	0.34762	0.92906
40698_at	X96719	C-type (calcium dependent, carbohydrate-recognition domain) lectin, superfamily member 2 (activation-induced)	0.09360	0.92811
36792_at	Z24727	tropomyosin 1 (alpha)	0.99798	0.92748

671_at	J03040	secreted protein, acidic, cysteine-rich (osteonectin)	0.39330	0.92399
36533_at	D83402	prostaglandin I2 (prostacyclin) synthase	0.77145	0.92383
39690_at	X97671	alpha-actinin-2-associated LIM protein	0.69538	0.92217
1106_s_at	M12959	T cell receptor alpha locus	0.12654	0.92215
37023_at	J02923	lymphocyte cytosolic protein 1 (L-plastin)	0.59217	0.92173
38454_g_at	X15606	intercellular adhesion molecule 2	0.07295	0.92090
39814_s_at	AI052724	retinal short-chain dehydrogenase/reductase 4	0.97797	0.91142
AFFX-BioB-M_at	J04423	NA	0.24036	0.91054
33328_at	W28612	NA	0.30594	0.90977
1596_g_at	L06139	TEK tyrosine kinase, endothelial (venous malformations, multiple cutaneous and mucosal)	0.53476	0.90756
34644_at	AB021288	beta-2-microglobulin	0.54540	0.90168
36790_at	M19267	tropomyosin 1 (alpha)	0.01332	0.89796
770_at	D00632	glutathione peroxidase 3 (plasma)	0.39374	0.89785
36239_at	Z49194	POU domain, class 2, associating factor 1	0.30462	0.89532
33440_at	U19969	transcription factor 8 (represses interleukin 2 expression)	0.75506	0.89436
38057_at	AL049798	dermatopontin	0.08318	0.89354
38319_at	AA919102	CD3D antigen, delta polypeptide (TiT3 complex)	0.97716	0.89335
AFFX-BioC-3_at	J04423	NA	0.29338	0.88838
35872_at	D50640	phosphodiesterase 3B, cGMP-inhibited	0.36364	0.88657
41738_at	M64110	caldesmon 1	0.93779	0.88126
31855_at	U61374	sushi-repeat-containing protein, X-linked	0.46189	0.88085
38006_at	M37766	CD48 antigen (B-cell membrane protein)	0.60797	0.88034
39593_at	AI432401	fibrinogen-like 2	0.24127	0.87936
40775_at	AL021786	integral membrane protein 2A	0.19208	0.87898
33113_at	U65093	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	0.11774	0.87351
35681_r_at	AB011141	zinc finger homeobox 1b	0.73793	0.87238
36577_at	Z24725	pleckstrin homology domain containing, family C (with FERM domain) member 1	0.93442	0.86990
297_g_at	X79535	tubulin, beta polypeptide	0.75944	0.86942
428_s_at	V00567	beta-2-microglobulin	0.48596	0.86863
35792_at	U67963	monoglyceride lipase	0.12384	0.86721
40019_at	M60830	ecotropic viral integration site 2B	0.68539	0.85520
37604_at	U44111	histamine N-methyltransferase	0.77680	0.85382
647_at	L35545	protein C receptor, endothelial (EPCR)	0.25995	0.85366
36629_at	AI635895	delta sleep inducing peptide, immunoreactor	0.83372	0.85247
40607_at	U97105	dihydropyrimidinase-like 2	0.46060	0.85074
34407_at	U77594	retinoic acid receptor responder (tazarotene induced) 2	0.72896	0.84738
39994_at	D10925	chemokine (C-C motif) receptor 1	0.42855	0.84580
37403_at	X05908	annexin A1	0.28166	0.84437
40480_s_at	M14333	FYN oncogene related to SRC, FGR, YES	0.72578	0.84119
35717_at	AB020629	ATP-binding cassette, sub-family A (ABC1), member 8	0.61682	0.83843
39470_at	AL049974	NA	0.76538	0.83821
39674_r_at	X97671	extracellular matrix protein 2, female organ and adipocyte specific	0.35367	0.83716
268_at	L34657	platelet/endothelial cell adhesion molecule (CD31 antigen)	0.80953	0.83540
581_at	M61916	laminin, beta 1	0.96636	0.83503
40063_at	U22897	nuclear domain 10 protein	0.56927	0.83151
33243_at	AF099935	TNF-induced protein	0.94215	0.83130
40399_r_at	X69878	mesenchyme homeo box 2 (growth arrest-specific homeo box)	0.17933	0.82724
35985_at	AB023137	A kinase (PRKA) anchor protein 2	0.81831	0.82505
33705_at	L20971	phosphodiesterase 4B, cAMP-specific (phosphodiesterase E4 dunce homolog, Drosophila)	0.89911	0.82439
35435_s_at	AF001903	L-3-hydroxyacyl-Coenzyme A dehydrogenase, short chain	0.66616	0.82415
36156_at	U41518	aquaporin 1 (channel-forming integral protein, 28kDa)	0.09910	0.82258
AFFX-BioC-5_at	J04423	NA	0.81549	0.82119
39174_at	X77548	nuclear receptor coactivator 4	0.53537	0.82056
37573_at	AF007150	angiopoietin-like 2	0.49852	0.81946

595_at	M59465	tumor necrosis factor, alpha-induced protein 3	0.14558	0.81468
39799_at	M94856	fatty acid binding protein 5 (psoriasis-associated)	0.43247	0.81291
35649_at	U80055	cysteine dioxygenase, type I	0.14954	0.81248
37598_at	Z84718	Ras association (RalGDS/AF-6) domain family 2	0.83524	0.80873
37688_f_at	M31932	Fc fragment of IgG, low affinity IIa, receptor for (CD32)	0.24103	0.80813
32067_at	S68271	cAMP responsive element modulator	0.94492	0.80744
31508_at	S73591	thioredoxin interacting protein	0.18059	0.80713
36874_at	M26004	complement component (3d/Epstein Barr virus) receptor 2	0.67153	0.80349
1709_g_at	U07620	mitogen-activated protein kinase 10	0.37241	0.80319
39372_at	W26480	fatty acid desaturase 1	0.34998	0.80307
40978_s_at	X56468	cerebellar degeneration-related protein 1, 34kDa	0.92176	0.80274
37399_at	Z84718	aldo-keto reductase family 1, member C3 (3-alpha hydroxysteroid dehydrogenase, type II)	0.24018	0.80111
39839_at	M24069	cold shock domain protein A	0.41781	0.80096
39760_at	AL031781	quaking homolog, KH domain RNA binding (mouse)	0.21344	0.80056
39317_at	D86324	cytidine monophosphate-N-acetylneuraminic acid hydroxylase (CMP-N-acetylneuraminate monooxygenase)	0.69175	0.80056
1478_at	L10717	IL2-inducible T-cell kinase	0.19676	0.79901
34183_at	AL080169	DKFZP434C171 protein	0.90779	0.79830
40509_at	J04058	electron-transfer-flavoprotein, alpha polypeptide (glutaric aciduria II)	0.09221	0.79652
33803_at	J02973	thrombomodulin	0.48197	0.79538
37637_at	U27655	regulator of G-protein signalling 3	0.92563	0.79527
37233_at	AF079167	oxidised low density lipoprotein (lectin-like) receptor 1	0.22272	0.79457
39081_at	AI547258	metallothionein 2A	0.39656	0.79255
37009_at	AL035079	catalase	0.52818	0.79234
41530_at	D16294	acetyl-Coenzyme A acyltransferase 2 (mitochondrial 3-oxoacyl-Coenzyme A thiolase)	0.80070	0.79163
38420_at	Y14690	collagen, type V, alpha 2	0.29804	0.79034
583_s_at	M30257	vascular cell adhesion molecule 1	0.50438	0.79014
32249_at	M65292	H factor (complement)-like 1	0.77091	0.78892
37394_at	J03507	complement component 7	0.88468	0.78842
34853_at	AB007865	fibronectin leucine rich transmembrane protein 2	0.80388	0.78727
41237_at	D32129	major histocompatibility complex, class I, A	0.28894	0.78696
36650_at	D13639	cyclin D2	0.87683	0.78594
40471_at	Y09048	peroxisomal farnesylated protein	0.16642	0.78421
41454_at	W27949	heme binding protein 2	0.89897	0.78331
39781_at	U20982	insulin-like growth factor binding protein 4	0.90959	0.78282
36878_f_at	M60028	major histocompatibility complex, class II, DQ beta 1	0.56955	0.78267
37200_at	J04162	Fc fragment of IgG, low affinity IIIa, receptor for (CD16)	0.43079	0.78199
34820_at	M57399	pleiotrophin (heparin binding growth factor 8, neurite growth-promoting factor 1)	0.89579	0.78032
36053_at	AF041248	cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	0.93854	0.77941
32112_s_at	AI800499	absent in melanoma 1	0.92581	0.77936
37025_at	AL120815	lipopolysaccharide-induced TNF factor	0.74421	0.77875
37624_at	M29458	carbonic anhydrase III, muscle specific	0.05846	0.77732
37397_at	L34657	platelet/endothelial cell adhesion molecule (CD31 antigen)	0.29506	0.77704
31438_s_at	Z22971	CD163 antigen	0.26524	0.77696
37623_at	X75918	nuclear receptor subfamily 4, group A, member 2	0.92410	0.77515
32363_at	AF059214	cholesterol 25-hydroxylase	0.52167	0.77404
AFFX-BioB-5_at	J04423	NA	0.61173	0.77271
37024_at	AF010312	lipopolysaccharide-induced TNF factor	0.64247	0.77229
33849_at	U02020	pre-B-cell colony-enhancing factor	0.55197	0.77117
34235_at	AB018301	G protein-coupled receptor 116	0.33874	0.76857
33499_s_at	M36612	hypothetical protein MGC27165	0.78312	0.76763
40079_at	AA156240	retinoic acid induced 3	0.84259	0.76667
39567_at	AB006190	aquaporin 7	0.79193	0.76611
38458_at	L39945	cytochrome b-5	0.60312	0.76508
41433_at	M73255	vascular cell adhesion molecule 1	0.16200	0.76489
38893_at	X80907	colony stimulating factor 2 receptor, beta, low-affinity (granulocyte-macrophage)	0.75044	0.76451
37310_at	X02419	plasminogen activator, urokinase	0.14086	0.76289

37493_at	H04668	colony stimulating factor 2 receptor, beta, low-affinity (granulocyte-macrophage)	0.44555	0.76163
39691_at	X97671	SH3-domain GRB2-like endophilin B1	0.62041	0.76018
37543_at	D25304	Rac/Cdc42 guanine nucleotide exchange factor (GEF) 6	0.06580	0.75901
160029_at	X07109	protein kinase C, beta 1	0.90609	0.75656
41839_at	L13698	growth arrest-specific 1	0.75905	0.75577
36980_at	U03105	proline-rich nuclear receptor coactivator 1	0.74753	0.75531
38796_at	X03084	complement component 1, q subcomponent, beta polypeptide	0.59629	0.75407
39338_at	AI201310	S100 calcium binding protein A10 (annexin II ligand, calpactin I, light polypeptide (p11))	0.74818	0.75386
37219_at	X72755	chemokine (C-X-C motif) ligand 9	0.31991	0.75180
38578_at	M63928	tumor necrosis factor receptor superfamily, member 7	0.38356	0.75052
36674_at	J04130	chemokine (C-C motif) ligand 4	0.64903	0.74902
38774_at	U77942	syntaxin 7	0.88811	0.74894
37391_at	X12451	cathepsin L	0.29832	0.74864
1147_at	X12795	nuclear receptor subfamily 2, group F, member 1	0.55784	0.74829
1761_at	D37965	platelet-derived growth factor receptor-like	0.39293	0.74760
40749_at	X07203	membrane-spanning 4-domains, subfamily A, member 1	0.52601	0.74672
36979_at	M20681	solute carrier family 2 (facilitated glucose transporter), member 3	0.54528	0.74617
AFFX-HSAC07/X00351_3_st	X00351	actin, beta	0.18568	0.74588
36159_s_at	U29185	prion protein (p27-30) (Creutzfeld-Jakob disease, Gerstmann-Strausler-Scheinker syndrome, fatal familial insomnia)	0.24095	0.74470
41871_at	AI660929	lung type-I cell membrane-associated glycoprotein	0.69013	0.73979
38026_at	U01244	fibulin 1	0.37915	0.73944
36635_at	AB023173	ATPase, Class VI, type 11B	0.41069	0.73838
38745_at	X76488	lipase A, lysosomal acid, cholesterol esterase (Wolman disease)	0.42809	0.73745
35012_at	M81750	myeloid cell nuclear differentiation antigen	0.36484	0.73706
33500_i_at	S71043	immunoglobulin heavy constant mu	0.84541	0.73669
37707_i_at	M81118	alcohol dehydrogenase 5 (class III), chi polypeptide	0.39630	0.73550
41755_at	AB023194	KIAA0977 protein	0.93761	0.73513
36656_at	M98399	CD36 antigen (collagen type I receptor, thrombospondin receptor)	0.17093	0.73254
2036_s_at	M59040	CD44 antigen (homing function and Indian blood group system)	0.31270	0.73123
38077_at	X52022	collagen, type VI, alpha 3	0.73445	0.73037
41505_r_at	AF055376	v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)	0.48056	0.72973
35410_at	U81234	chemokine (C-X-C motif) ligand 6 (granulocyte chemotactic protein 2)	0.94371	0.72808
227_g_at	M33336	protein kinase, cAMP-dependent, regulatory, type I, alpha (tissue specific extinguisher 1)	0.29303	0.72703
36773_f_at	M81141	major histocompatibility complex, class II, DQ beta 1	0.36558	0.72696
36659_at	X05610	collagen, type IV, alpha 2	0.50209	0.72574
40767_at	M59499	tissue factor pathway inhibitor (lipoprotein-associated coagulation inhibitor)	0.83878	0.72385
37383_f_at	X58536	major histocompatibility complex, class I, C	0.34433	0.72299
33305_at	M93056	serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 1	0.62059	0.72142
39822_s_at	AF078077	growth arrest and DNA-damage-inducible, beta	0.22866	0.71960
38374_at	AF050110	TGFB inducible early growth response	0.48733	0.71756
31897_at	D64142	downregulated in ovarian cancer 1	0.86622	0.71664
33809_at	AL049933	guanine nucleotide binding protein (G protein), alpha inhibiting activity polypeptide 1	0.62795	0.71438
34210_at	N90866	CDW52 antigen (CAMPATH-1 antigen)	0.90640	0.71168
37684_at	AB020687	solute carrier family 21 (organic anion transporter), member 9	0.22353	0.71162
1323_at	X04803	ubiquitin B	0.25016	0.71151

32689_s_at	D86096	prostaglandin E receptor 3 (subtype EP3)	0.54627	0.71026
38768_at	X96752	L-3-hydroxyacyl-Coenzyme A dehydrogenase, short chain	0.44397	0.70748
36889_at	M33195	Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide	0.87477	0.70726
36753_at	AF072099	leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 4	0.21325	0.70714
33834_at	L36033	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	0.65288	0.70577
38320_s_at	L11706	lipase, hormone-sensitive	0.85459	0.70553
39864_at	D78134	cold inducible RNA binding protein	0.03460	0.70520
41747_s_at	U49020	MADS box transcription enhancer factor 2, polypeptide A (myocyte enhancer factor 2A)	0.82787	0.70229
1461_at	M69043	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	0.26793	0.70224
37017_at	M22430	phospholipase A2, group IIA (platelets, synovial fluid)	0.40704	0.70055
1368_at	M27492	interleukin 1 receptor, type I	0.48205	0.70039
37747_at	U05770	annexin A5	0.30298	0.69915
767_at	AF001548	myosin, heavy polypeptide 11, smooth muscle	0.98159	0.69879
33730_at	AF095448	retinoic acid induced 3	0.13372	0.69718
37043_at	AL021154	E2F transcription factor 2	0.62130	0.69715
39640_at	X97671	glutamine-fructose-6-phosphate transaminase 2	0.30512	0.69628
1062_g_at	U00672	interleukin 10 receptor, alpha	0.61732	0.69585
39959_at	AL031983	gamma-aminobutyric acid (GABA) B receptor, 1	0.91080	0.69518
41422_at	D78011	dihydropyrimidinase	0.29684	0.69469
32424_at	D84424	hyaluronan synthase 1	0.20338	0.69396
33790_at	AI720438	chemokine (C-C motif) ligand 15	0.83255	0.69263
41609_at	U15085	major histocompatibility complex, class II, DM beta	0.40963	0.69210
AFFX-CreX-5_at	X03453	NA	0.29783	0.69181
36711_at	AL021977	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian)	0.26017	0.69127
41225_at	AL049417	dual specificity phosphatase 3 (vaccinia virus phosphatase VH1-related)	0.06542	0.69117
590_at	M32334	intercellular adhesion molecule 2	0.09082	0.69055
38826_at	D50918	septin 6	0.62091	0.69032
35752_s_at	M15036	protein S (alpha)	0.14335	0.68997
37983_at	S77410	angiotensin II receptor, type 1	0.17657	0.68889
1491_at	M31166	pentaxin-related gene, rapidly induced by IL-1 beta	0.16336	0.68852
37078_at	J04132	CD3Z antigen, zeta polypeptide (TiT3 complex)	0.55008	0.68760
34378_at	X97324	adipose differentiation-related protein	0.74773	0.68751
36908_at	M93221	mannose receptor, C type 1	0.99474	0.68687
36130_f_at	R92331	metallothionein 1E (functional)	0.22509	0.68636
33813_at	AI813532	tumor necrosis factor receptor superfamily, member 1B	0.97424	0.68581
39091_at	AF070523	cytoskeleton related vitamin A responsive protein	0.59294	0.68568
35832_at	AB029000	sulfatase 1	0.97249	0.68558
661_at	L13698	growth arrest-specific 1	0.19500	0.68417
41439_at	AJ001381	myosin IB	0.38664	0.68316
39397_at	M64497	nuclear receptor subfamily 2, group F, member 2	0.04674	0.68183
36892_at	AF032108	integrin, alpha 7	0.59819	0.67952
36503_at	AB002409	chemokine (C-C motif) ligand 21	0.35965	0.67922
174_s_at	U59325	intersectin 2	0.40345	0.67828
AFFX-BioB-3_at	J04423	NA	0.85266	0.67802
35331_at	U97067	catenin (cadherin-associated protein), alpha-like 1	0.48925	0.67779
38254_at	AB020689	KIAA0882 protein	0.21533	0.67762
36280_at	U26174	granzyme K (serine protease, granzyme 3; tryptase II)	0.75900	0.67748
159_at	U40992	vascular endothelial growth factor C	0.48649	0.67659
1377_at	M58603	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	0.41563	0.67559
34198_at	U12128	protein tyrosine phosphatase, non-receptor type 13 (APO-1/CD95 (Fas)-associated phosphatase)	0.15005	0.67549
40522_at	X59834	glutamate-ammonia ligase (glutamine synthase)	0.79914	0.67515
35261_at	W07033	glia maturation factor, gamma	0.48414	0.67215

41124_r_at	L35594	ectonucleotide pyrophosphatase/phosphodiesterase 2 (autotaxin)	0.54091	0.67179
31575_f_at	M14087	NA	0.45839	0.67158
33943_at	L20941	ferritin, heavy polypeptide 1	0.54136	0.66974
1767_s_at	X14885	transforming growth factor, beta 3	0.79004	0.66900
AFFX-BioDn-3_at	J04423	NA	0.42264	0.66798
39069_at	AF053944	AE binding protein 1	0.13066	0.66791
376_at	Z84718	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C	0.73801	0.66361
610_at	M15169	adrenergic, beta-2-, receptor, surface	0.26832	0.66313
37892_at	J04177	collagen, type XI, alpha 1	0.28326	0.66131
38995_at	AF000959	claudin 5 (transmembrane protein deleted in velocardiofacial syndrome)	0.79053	0.66095
41257_at	D16217	calpastatin	0.33747	0.66029
34760_at	D14664	type I transmembrane C-type lectin receptor DCL-1	0.45971	0.65662
37148_at	AF025533	leukocyte immunoglobulin-like receptor, subfamily B (with TM and ITIM domains), member 3	0.52118	0.65620
37294_at	X61123	B-cell translocation gene 1, anti-proliferative	0.20484	0.65533
37542_at	D86961	lipoma HMGIC fusion partner-like 2	0.74916	0.65180
837_s_at	U43944	malic enzyme 1, NADP(+)-dependent, cytosolic	0.99374	0.65128
33501_r_at	S71043	immunoglobulin heavy constant mu	0.97950	0.64949
35790_at	AF054179	vacuolar protein sorting 26 (yeast)	0.94219	0.64923
38833_at	X00457	major histocompatibility complex, class II, DP alpha 1	0.40055	0.64820
41531_at	AI445461	transmembrane 4 superfamily member 1	0.22557	0.64817
37645_at	Z22576	CD69 antigen (p60, early T-cell activation antigen)	0.39886	0.64667
39409_at	M14058	complement component 1, r subcomponent	0.18833	0.64537
33390_at	AA203487	serine/threonine kinase 17b (apoptosis-inducing)	0.40573	0.64442
36976_at	D21255	cadherin 11, type 2, OB-cadherin (osteoblast)	0.90470	0.64311
32091_at	AB007915	KIAA0446 gene product	0.16846	0.64302
245_at	M25280	selectin L (lymphocyte adhesion molecule 1)	0.55771	0.64229
33830_at	AW026535	leptin receptor	0.69626	0.64228
35776_at	AF064243	intersectin 1 (SH3 domain protein)	0.58109	0.64136
37420_i_at	AL022723	major histocompatibility complex, class I, F	0.73519	0.64066
37544_at	X64318	nuclear factor, interleukin 3 regulated	0.35854	0.63987
216_at	M98539	prostaglandin D2 synthase 21kDa (brain)	0.37453	0.63808
38666_at	M85169	pleckstrin homology, Sec7 and coiled-coil domains 1(cytohesin 1)	0.76049	0.63776
38323_at	AC005162	carboxypeptidase, vitellogenic-like	0.97692	0.63717
40575_at	AB011155	discs, large homolog 5 (Drosophila)	0.59008	0.63636
37692_at	AI557240	diazepam binding inhibitor (GABA receptor modulator, acyl-Coenzyme A binding protein)	0.39246	0.63501
41000_at	U68723	checkpoint suppressor 1	0.95874	0.63487
36030_at	AL080214	HOM-TES-103 tumor antigen-like	0.50402	0.63487
35267_g_at	AL049288	bladder cancer associated protein	0.49798	0.63435
32184_at	X61118	LIM domain only 2 (rhombotin-like 1)	0.46845	0.63283
37459_at	X57527	collagen, type VIII, alpha 1	0.58842	0.63278
36515_at	AJ238764	UDP-N-acetylglucosamine-2-epimerase/N-acetylmannosamine kinase	0.38986	0.63244

Symbol	probe-id	Acc ID	Full Name
ADN	40282_s_at	M84256	adipsin
AGRN	33454_at	AF016903	agrin
BF	35822_at	L15702	B-factor
CD24	266_at	L33930	CD24 antigen
CD9	39389_at	M38690	CD9 antigen
CLDN3	33904_at	AB000714	claudin 3
CLDN4	35276_at	AB000712	claudin 4
CP	39008_at	M13699	ceruloplasmin
EZH2	37305_at	U61145	enhancer of zeste homolog 2
FHL2	38422_at	U29332	four and a half LIM domains 2
FOXO1A	40570_at	AF032885	forkhead box O1A
HE4	33933_at	X63187	WAP four-disulfide core domain 2
HPN	37639_at	X07732	hepsin
IFI-15K	1107_at	M13755	interferon, alpha-inducible protein, 15kDa
IGL	33273_f_at	X57809	immunoglobulin lambda locus
KLK10	36838_at	AF055481	kallikrein 10
KLK11	40035_at	AB012917	kallikrein 11
KLK13	36406_at	AA401397	kallikrein 13
KLK2	217_at	S39329	kallikrein 2
KLK3	1804_at	X07730	kallikrein 3
KLK6	37554_at	U62801	kallikrein 6
KLK7	38143_at	L33404	kallikrein 7
KLK8	37131_at	AB008390	kallikrein 8
KRT18	35766_at	M26326	keratin 18
KRT19	40899_at	Y00503	keratin 19
KRT8	33824_at	X74929	keratin 8
LMNB1	37985_at	L37747	lamin B1
LPL	41209_at	M15856	lipoprotein lipase
LU	40093_at	X83425	Lutheran blood group
OP	2092_at	J04675	osteopontin
OPCML	41093_at	AF070577	opioid binding protein/cell adhesion molecule like
PEG3	39701_at	AB006625	paternally expressed 3
PLIN	37122_at	AB005293	Perilipin
PRAME	157_at	U65011	preferentially expressed antigen of melanoma
PRSS8	634_at	L41351	protease, serine 8
PTTG1	40412_at	AF095288	pituitary tumour transforming 1
SAA1	33272_at	AA829286	serum amyloid A1
SCGB2A1	41066_at	NM_002407	secretoglobin, family 2A, member 1
SLPI	32275_at	X04470	secretory leukocyte protease inhibitor
TACSTD2	575_s_at	J04152	tumour-associated calcium signal transducer 2
VEGF	36100_at	AF024710	vascular endothelial growth factor
WISP2	35898_at	AF100780	WNT1 inducible signalling pathway protein 2

Table 3.3. Summary of the names and abbreviations of genes discussed.
Symbol here is the most commonly used abbreviation, usually from OMIM (Online Mendelian Inheritance in Man). Probe is the unique Affymetrix probe ID. The Accession is the NCBI Refseq, or reference sequence for this gene.

3.6 Real-Time Quantitative Reverse Transcription PCR (QTR-PCR)

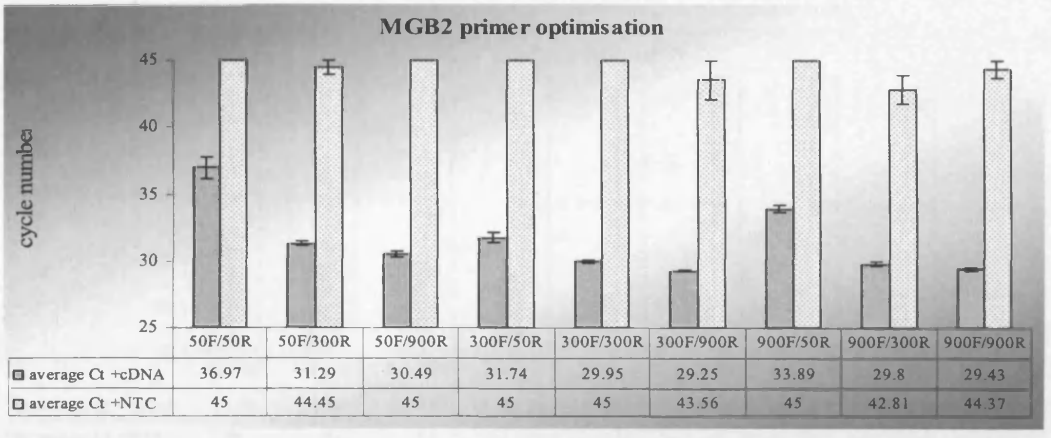
3.6.1 Primer Optimisation

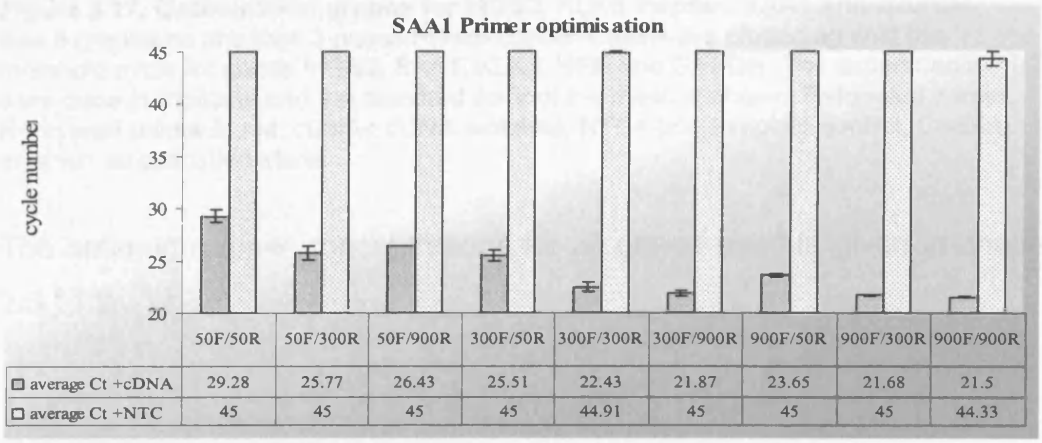
In order to perform real-time qRT-PCR, forward and reverse primers were chosen for each gene (see section 2.5.6.2). Primer optimisation for all genes was based on varying forward and reverse primers. Every combination was run in triplicate with and without template (non-template control, NTC) and with the PCR thermal cycle conditions described in Table 2.6.

Reverse primer (nM)	Forward primer (nM)		
	50	300	900
50	50/50	50/300	50/900
300	300/50	300/300	300/900
900	900/50	900/300	900/900

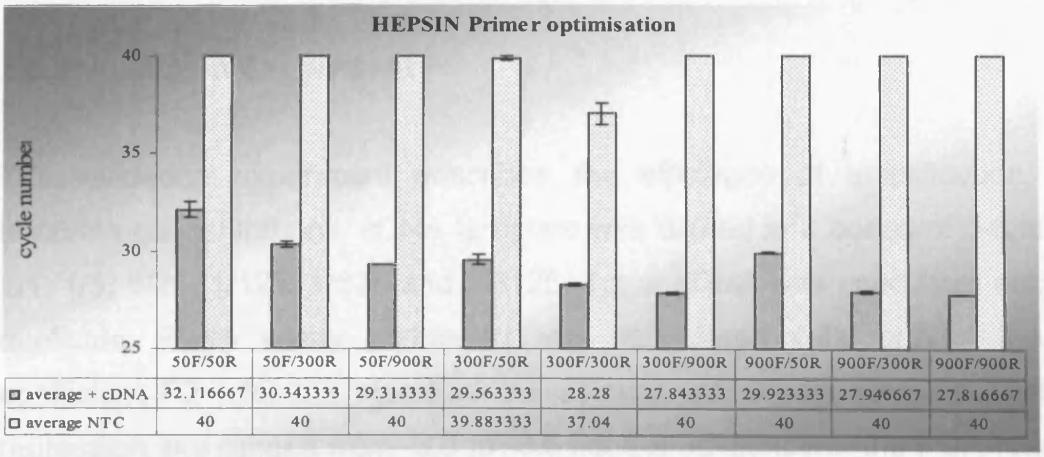
Table 3.4. Primer concentrations used in the primer optimisation matrix.
Nine different combinations were used for each optimisation (modified from the Taqman® Universal PCR Master Mix protocol).

The optimal performance is achieved by selecting the primer concentrations that provide the lowest threshold cycle (CT) for a fixed amount of template and no amplification was observed in the NTC for all genes (Figure 3.17 below).

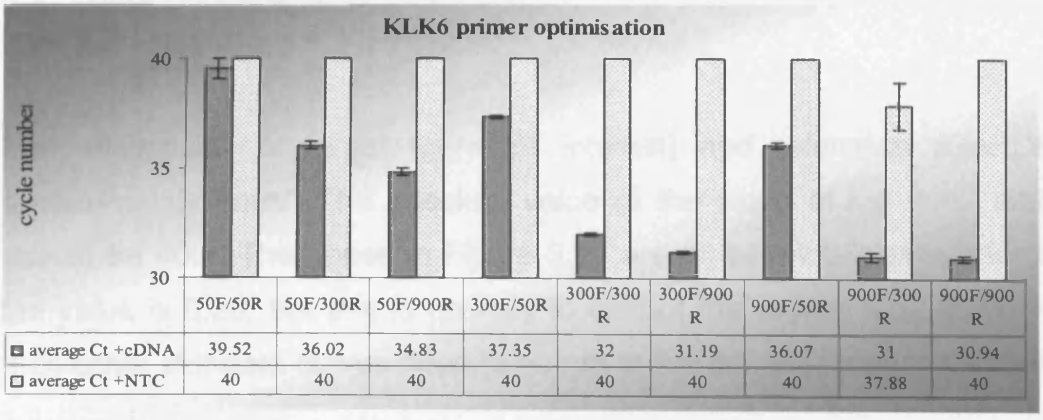




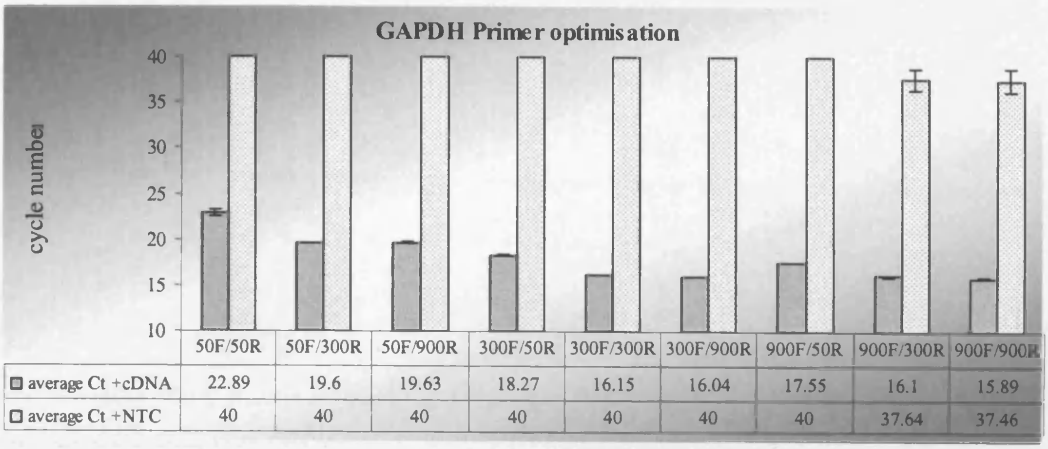
	50F/50R	50F/300R	50F/900R	300F/50R	300F/300R	300F/900R	900F/50R	900F/300R	900F/900R
average Ct +cDNA	29.28	25.77	26.43	25.51	22.43	21.87	23.65	21.68	21.5
average Ct +NTC	45	45	45	45	44.91	45	45	45	44.33



	50F/50R	50F/300R	50F/900R	300F/50R	300F/300R	300F/900R	900F/50R	900F/300R	900F/900R
average + cDNA	32.116667	30.343333	29.313333	29.563333	28.28	27.843333	29.923333	27.946667	27.816667
average NTC	40	40	40	39.883333	37.04	40	40	40	40



	50F/50R	50F/300R	50F/900R	300F/50R	300F/300R	300F/900R	900F/50R	900F/300R	900F/900R
average Ct +cDNA	39.52	36.02	34.83	37.35	32	31.19	36.07	31	30.94
average Ct +NTC	40	40	40	40	40	40	40	37.88	40



	50F/50R	50F/300R	50F/900R	300F/50R	300F/300R	300F/900R	900F/50R	900F/300R	900F/900R
average Ct +cDNA	22.89	19.6	19.63	18.27	16.15	16.04	17.55	16.1	15.89
average Ct +NTC	40	40	40	40	40	40	40	37.64	37.46

Figure 3.17. Optimisation graphs for MGB2, KLK6, hepsin, SAA1 and GAPDH. See 5 graphs on previous 2 pages.Primer concentrations are plotted against the threshold cycle for genes MGB2, SAA1, KLK6, HPN and GAPDH. The experiments were done in triplicate and the standard error of the mean is shown. F=forward primer, R=reverse primer in nM. cDNA= cDNA template, NTC= non-template control, Ct=time at which amplification starts.

The optimum primer concentrations for all genes used is given in chapter 2 section 2.5.11.2.

3.6.2 Validation Experiment

The validation experiment describes the efficiency of amplification at different template concentrations. cDNA template was diluted in a series of 5-fold dilutions at 1/1, 1/5, 1/25, 1/125, 1/625 and 1/3125. 1µl of cDNA was used from each dilution in duplicate. Every assay performed was considered valid only if the correlation coefficient R2 , for the standard curves was >0.98 and the slope values for the regression line ranged from -3.3 to -3.9 i.e. the efficiency of the PCR reaction was at least 80% (efficiency = 10(-1/slope) – 1 x 100).

The efficiencies of target (gene of interest) and reference (GAPDH) must be approximately equal. The absolute value of the slope of log input amount vs □Ct should be <0.2. The genes in Figure 3.18 are all below 0.2 except for SAA1 where the value is 0.28, but this is unlikely to disrupt the experiment. If the value is >0.2 then either standard curves need to be run or the primers need to be redesigned.

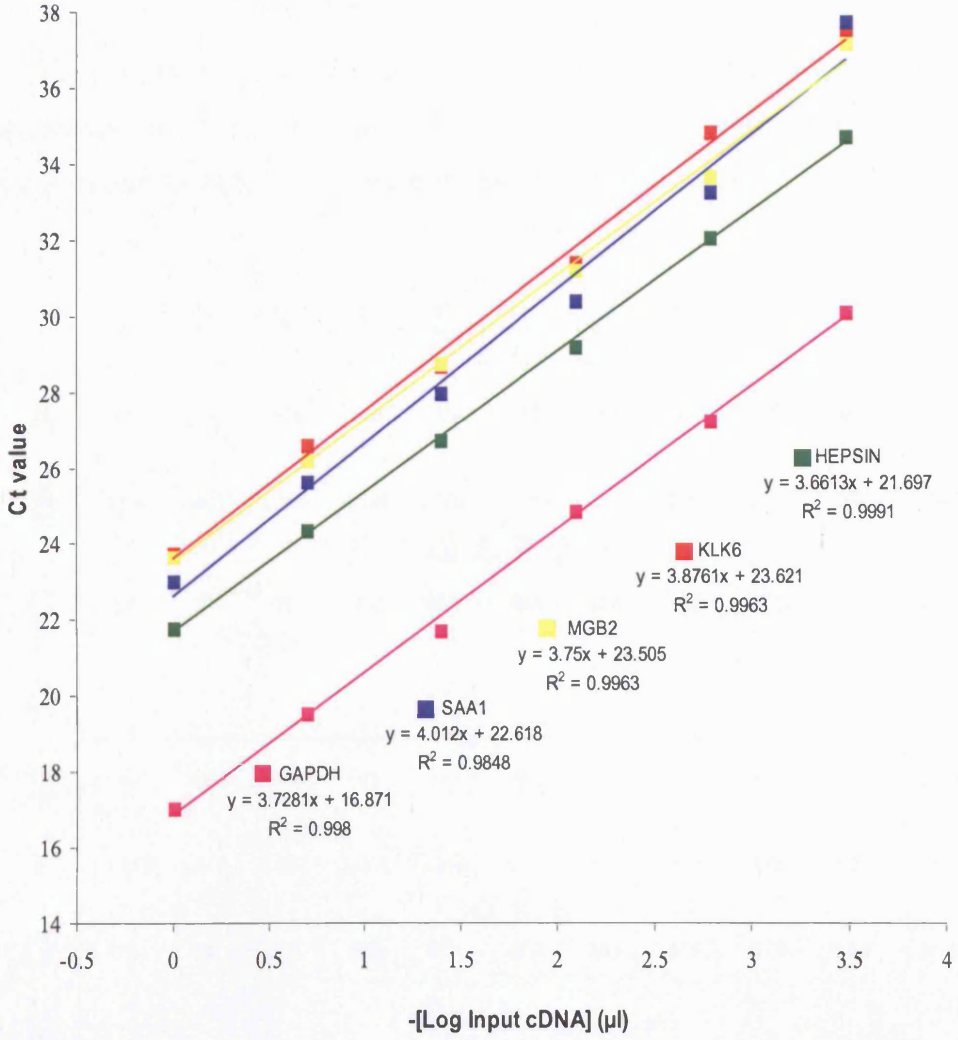


Figure 3.18. Ct validation experiment.

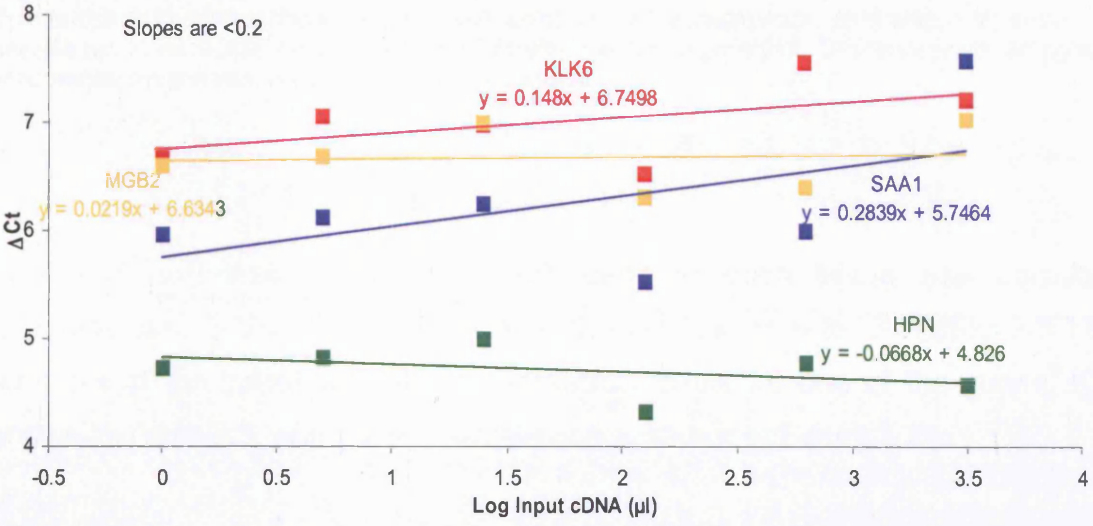


Figure 3.19. Relative efficiency plot

3.6.3 Expression Levels

After optimisation and validation experiments, the RNA levels for each gene were determined in all tissues (normal, LMP, primary and secondary cancers). The design of the experiment for each gene is shown in Figure 3.20.

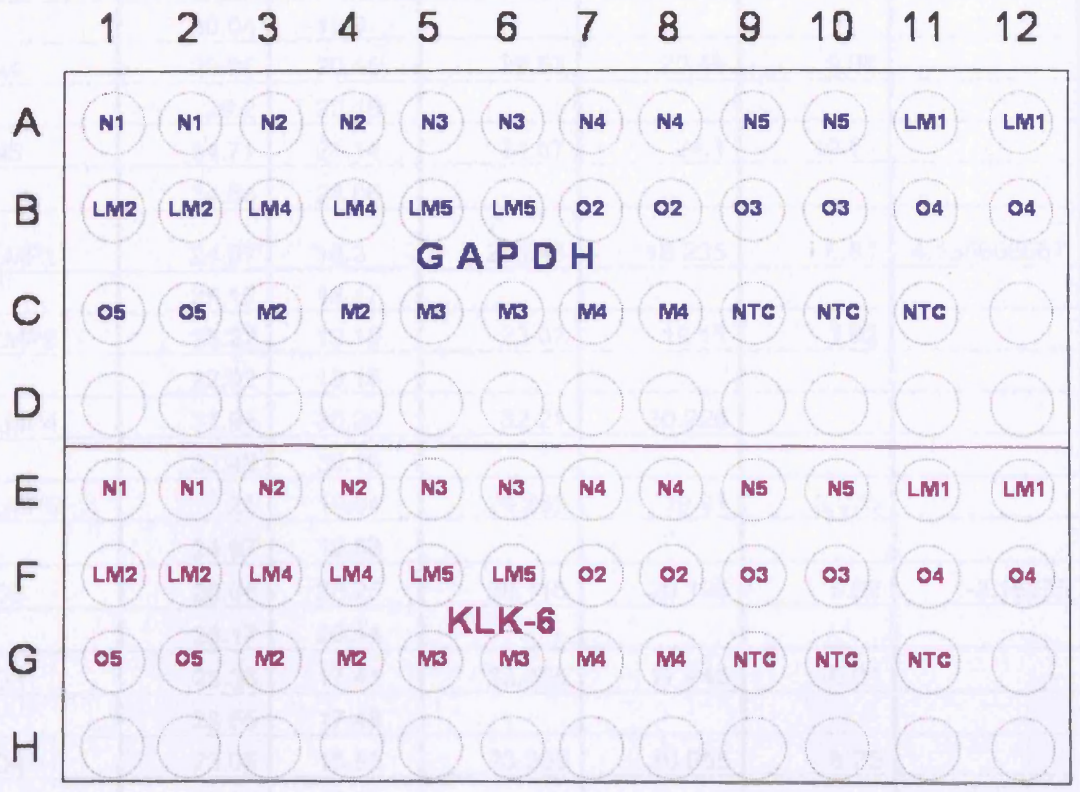


Figure 3.20. 96 well plate
This template illustrates the layout of cDNA samples for the expression level step, with each sample run in duplicate. N=normal ovary, LM=low malignant potential, O=primary ovarian cancer, M=omental metastasis, NTC=non-template control.

The RNA expression levels for each gene in each tissue was calculated by comparison to GAPDH levels in Normal tissues (see chapter 2 section 2.5.11.2). An example of the calculation of the expression levels for one of the genes, KLK6, is shown in Table 3.5, and the fold expression is shown in Figure 3.21.

Tissue SYBR Green	KLK6 Ct	GAPDH Ct	Average KLK6 Ct	Average GAPDH Ct	ΔCt	ΔΔCt	2^(-ΔΔCt)
N1	32.08	26.63	32.53	26.765	5.765	0	1
	32.98	26.9					
N2	31.79	19.73	31.415	19.94	11.475		
	31.04	20.15					
N3	30.08	19.96	30.06	19.93	10.13		
	30.04	19.9					
N4	29.86	20.45	29.53	20.45	9.08		
	29.2	20.45					
N5	34.71	24.14	34.67	24.1	10.57		
	34.63	24.06					
LMP1	24.97	18.3	25.045	18.235	6.81	-4.135666667	17.57760572
	25.12	18.17					
LMP2	23.22	19.15	23.07	19.15	3.92		
	22.92	19.15					
LMP4	31.94	30.26	32.21	30.225			
	32.48	30.19					
LMP5	25	19.94	24.985	19.91	5.075		
	24.97	19.88					
O2	26.06	20.25	26.115	20.195	5.92	-3.16275	8.955351131
	26.17	20.14					
O3	23.36	17.41	23.455	17.445	6.01		
	23.55	17.48					
O4	23.05	16.85	23.205	16.955	6.25		
	23.36	17.06					
O5	23.27	16.4	23.135	16.35	6.785		
	23	16.3					
M2	37.19	29.58	36.395	29.62		-1.644	3.125311521
	35.6	29.66					
M3	28.48	20.2	28.515	20.485	8.03		
	28.55	20.77					
M4	25.28	17.56	25.235	17.745	7.49		
	25.19	17.93					

Table 3.5. Relative quantitation of KLK6 in normal (N), low malignant potential (LMP), primary ovarian cancer (O) and omental secondaries (M).
ΔΔCt = ΔCt relative to Normal (ΔCt for KLK6 in normal ovary - ΔCt for KLK6 in LMP/cancer tissue. Ct = threshold cycle, ΔCt = Ct for KLK6 – Ct for GAPDH. 2^(-ΔΔCt) = measure of KLK6 over-expression relative to normal tissue.
The remaining genes, MGB2, SAA1 and HPN were calculated in the same fashion.

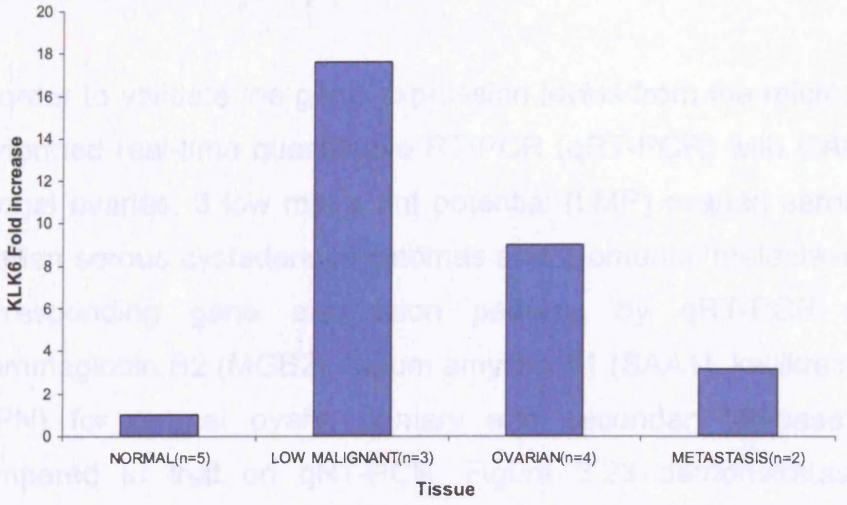


Figure 3.21. KLK6 fold expression based on above expression data (Table 3.5).

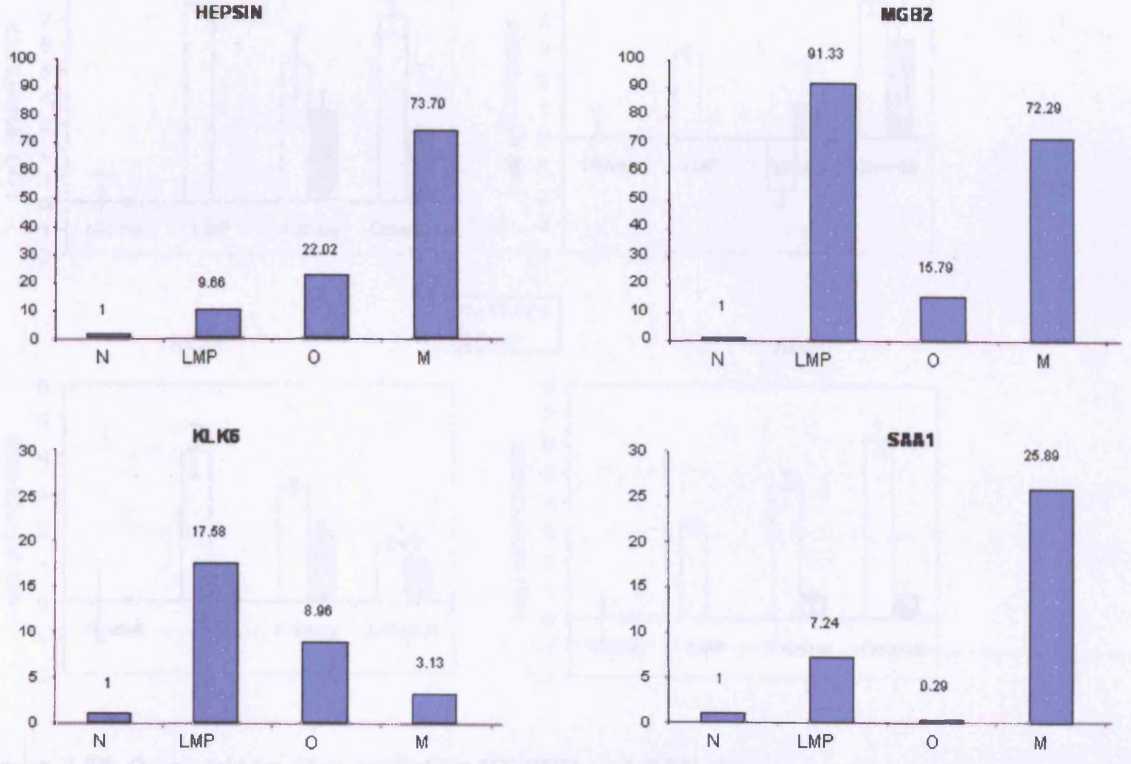


Figure 3.22. Fold expression of hepsin, MGB2, KLK6 and SAA1 in low malignant potential (LMP) tumour, primary ovarian cancer (O) and omental metastasis (M) relative to normal ovary.

3.6.4 Validation of Array Data with QRT-PCR

In order to validate the gene expression levels from the microarray experiments, we performed real-time quantitative RT-PCR (qRT-PCR) with GAPDH as a control in 5 normal ovaries, 3 low malignant potential (LMP) ovarian serous cancers, 4 primary ovarian serous cystadenocarcinomas and 2 omental metastases. Figure 3.22 shows corresponding gene expression patterns by qRT-PCR of the four genes, mammaglobin B2 (MGB2), serum amyloid A1 (SAA1), kallikrein-6 (KLK6) and hepsin (HPN) for normal ovary, primary and secondary disease on the microarrays compared to that on qRT-PCR. Figure 3.23 demonstrates that the differential expression pattern and the quantitative expression level of each of these four genes as determined by qRT-PCR were similar to those observed with the microarrays, confirming the reliability of our array expression data. Notably, qRT-PCR showed high expression of MGB2 and KLK6 in the LMP samples.

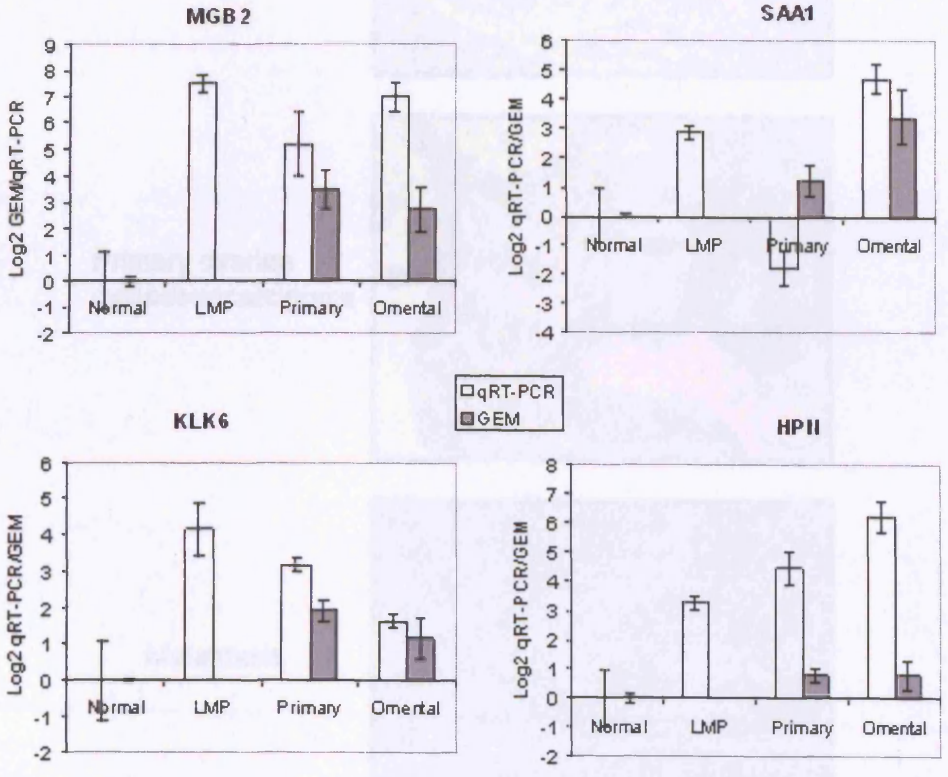


Figure 3.23. Comparison of quantitative RT-PCR and GEM data.
QRT-PCR data: (clear bars, Normal (n=5), Primary (n=5), LMP (n=3), and Metastasis (n=2)) and GEM data: (shaded bars, Normal (n=4), Primary (n=6), and Metastasis (n=6)) for MGB2, SAA1, KLK6, and HPN in normal, primary and omental metastasis samples. GEM data is in original Log2 scale, and qRT-PCR is single Log2 unit per round of amplification, error bars show the standard deviation. The Normal level is taken as a 0 baseline reference for both.

3.7 Immunohistochemistry

IHC was performed with one gene, hepsin. This prostate cancer serum biomarker was over-expressed in both primary ovarian cancer tissue and further over-expressed in omental tissue. There was staining of both normal ovarian surface epithelium (OSE) and malignant epithelial cells in primary cancer and omental metastasis. The pattern in malignant cells was distinct in being localised to the membrane. (Figure 3.24).

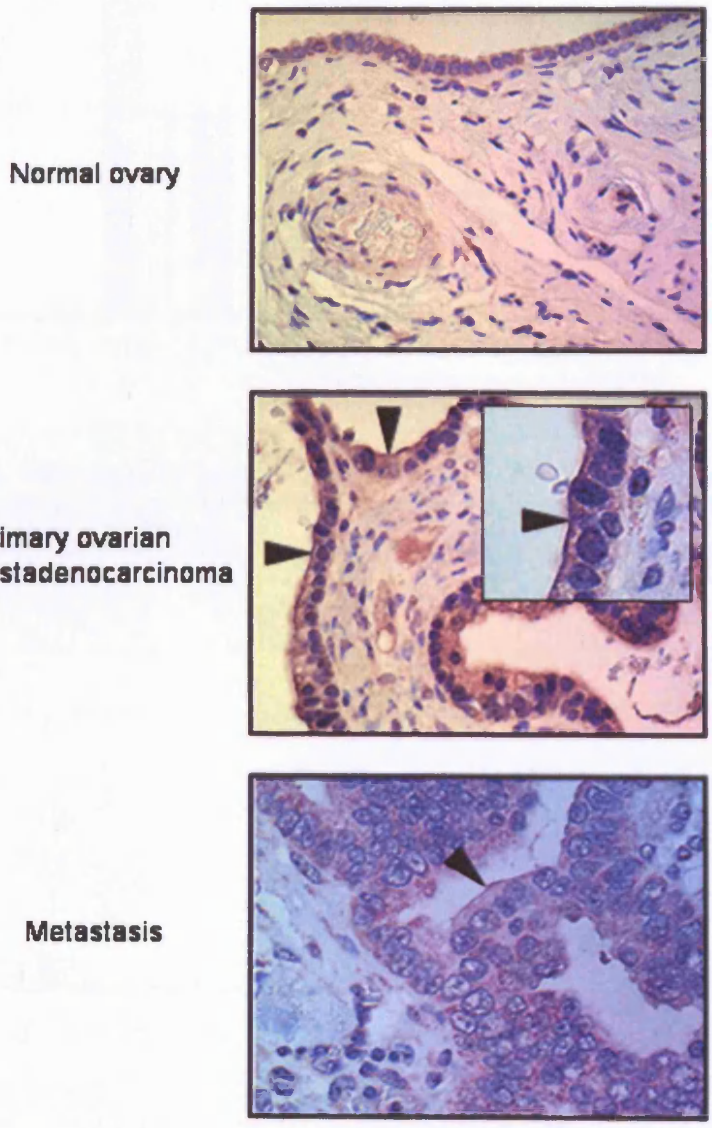


Figure 3.24. Immunohistochemical Staining for hepsin.
Hepsin stained normal and malignant epithelial cells. However, a prominent membrane staining (arrowheads) was only seen in malignant epithelial cells. Pictures 40x; Inset 100x.

3.8 Chemokine Receptor Expression In Ovarian Cancers

Semi-quantitative RT-PCR was performed to verify expression of chemokines in ovarian cancer. The most highly expressed chemokine in primary ovarian cancer is CXCR4 for both the semi-quantitative RT-PCR and the GEM data.

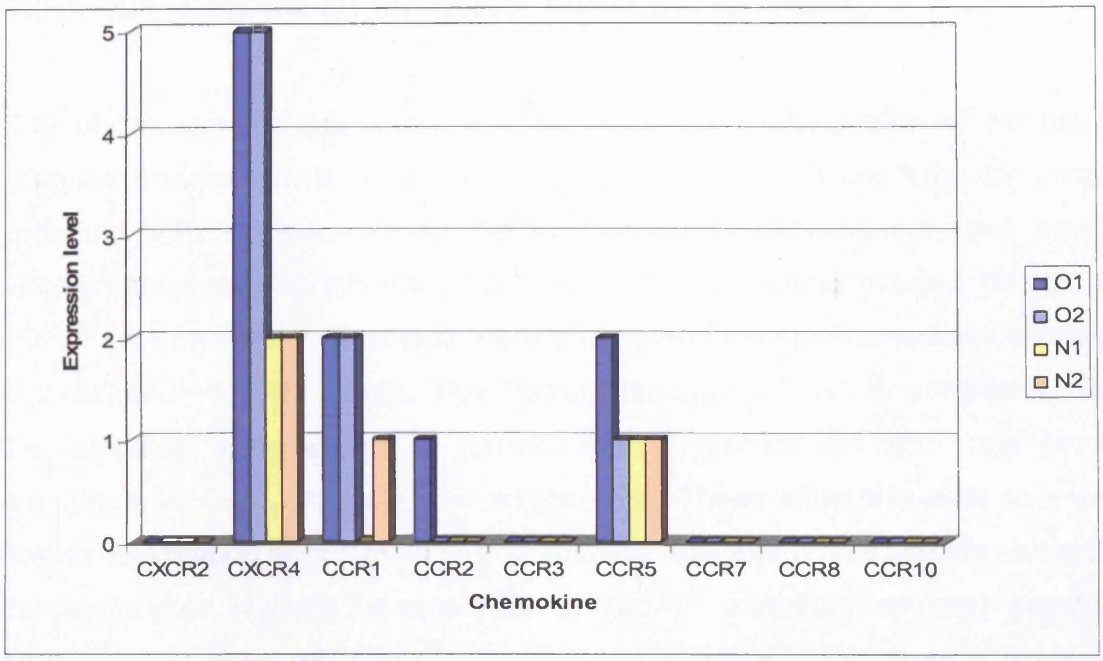


Figure 3.25. Semi-quantitative RT-PCR expression data for chemokines in ovarian cancer.
O1 &O2= 2 samples of primary ovarian cancer, N1 & N2= 2 samples of normal ovary. Expression levels are relative to GAPDH.

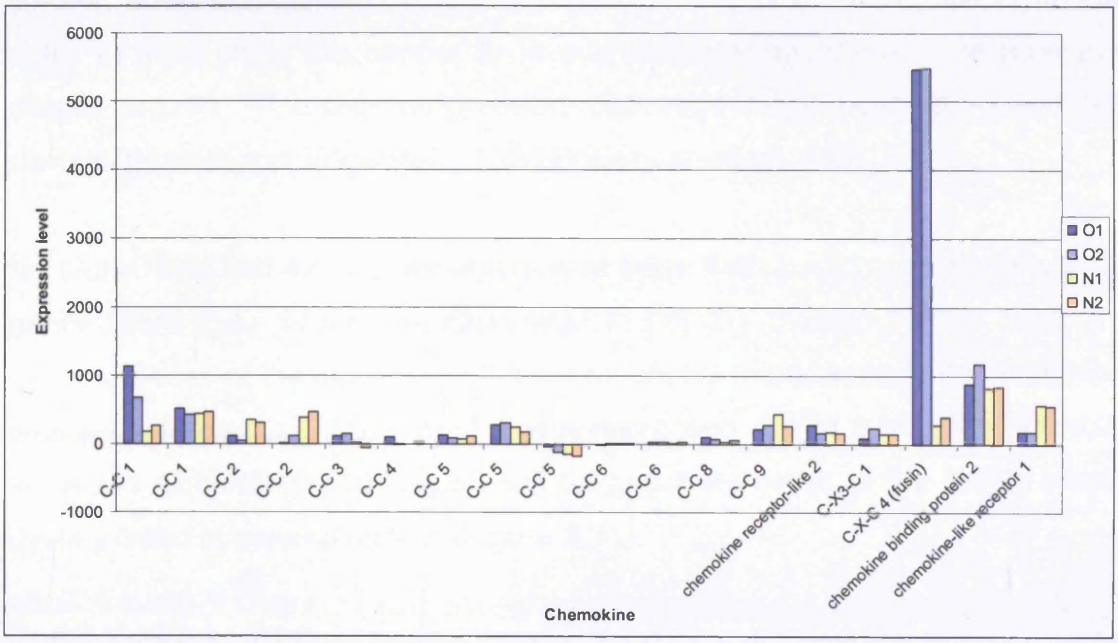


Figure 3.26. GEM data for chemokines in ovarian cancer.
O1 &O2= 2 samples of primary ovarian cancer, N1 & N2= 2 samples of normal ovary. Expression levels are relative to GAPDH.

CHAPTER 4

DISCUSSION

4.1 GEM Profile of Primary Ovarian Cancer

One of the aims of this study was to discover a GEM profile for serous ovarian adenocarcinoma which would allow us to understand more fully the progression pathway from normal ovarian tissue through primary cancer and then on to metastatic spread. To this end, the GEM profiles of normal ovaries, primary ovarian cancer and omental metastases were determined using oligonucleotide microarrays representing ~12,000 genes. The classic paradigm of cancer progression involves the multistep accumulation of genetic and epigenetic changes, and progressive alterations in gene expression in single cells. These allow the cells to escape the normal constraints of cell growth, and become malignant; they include self-sufficiency in proliferation signals, insensitivity to growth inhibitory signals, evasion from apoptosis, limitless replicative potential, and aberrant angiogenesis [Hanahan and Weinberg, 2000]. This enables a few malignant cells to form a clinically recognisable tumour mass. As the tumour grows, more genetic aberrations are accumulated in order to then allow the cancer to invade surrounding tissues and metastasise to distant organs. Multistep progression pathways have been proposed for most cancers [Kinzler and Vogelstein, 1996;Beckmann et al., 1997].

My study identified 421 genes which were more than 2-fold over-expressed and 118 genes more than 3-fold over-expressed in primary ovarian cancer than in normal ovaries. Some of these genes will have functional importance in carcinogenesis and disease progression; others will be over-expressed due to a reaction to the increase in growth stimulatory pathways. I will discuss here some of the genes identified as dysregulated in ovarian cancer (Figure 3.11).

In the epithelial markers cluster (Figure 3.11), claudin proteins are a large family of integral membrane proteins which play an important role in tight junction (TJ)

formation and function. Claudins-3 and -4 have been shown to be up-regulated in ovarian cancers and have been suggested as possible biomarkers and targets for therapy [Hough et al., 2001;Rangel et al., 2003]. Ovarian adenocarcinomas are characterized by initial local growth followed by spread into the peritoneal cavity at later stages of tumour progression. The cell-adhesion molecule E-cadherin (E-cad) plays an important role in maintaining tissue integrity. E-cadherin expression is rare in normal ovarian tissue but increases in benign and neoplastic ovarian epithelial tumours [Maines-Bandiera and Auersperg, 1997;Sundfeldt et al., 1997]. Secreted phosphoprotein 1 (SPP1), the gene encoding osteopontin (OP), is over-expressed in many human carcinomas [Agrawal et al., 2002;Hotte et al., 2002;Korkola et al., 2003]. OP is an integrin-binding protein and is involved in carcinogenesis and tumour spread.

In the cell division and growth cluster (Figure 3.11), cyclin D1 is over-expressed; this has previously been shown to be over-expressed in ovarian cancers and is related to prognosis [Diebold et al., 2000]. C-myc oncogene codes for a DNA binding protein which plays an important role in the regulation of cell growth. When over-expressed in tumours, it is involved in metastatic progression, and there is evidence that it is associated with poor prognosis in ovarian cancer [Wu et al., 2003], as well as cancers of the breast [Naidu et al., 2002] and prostate [Sato et al., 1999]. Lipocalin 2 and E2F transcription factor 3 have not previously been associated with ovarian cancer.

CD24 antigen (Figure 3.11) has been shown to be over-expressed in ovarian cancer and may have potential as a marker of poor prognosis [Kristiansen et al., 2002]. Hepsin is a serine protease and is present in the plasma membranes of most tissues, with highest expression in liver. Tanimoto et al noted increased expression in LMP and invasive ovarian tumours and concluded that hepsin is associated with the invasive process [Tanimoto et al., 1997]. Hepsin is also over-expressed in prostate cancers [Stephan et al., 2004;Dhanasekaran et al., 2001].

GEM profiling studies have reported several genes which are up- and down-regulated in ovarian cancers compared to normal ovaries. Table 4.1 lists a series of

22 up-regulated genes which have been identified by different studies and validated by independent methods such as Northern blot analysis or RT-PCR. These genes are only a few of the hundreds of genes which have been identified by GEM profiling. As these profiles represent gene expression, no information can be inferred regarding the function of these genes in the carcinogenic process. However, as various studies have identified the same genes, these represent a true biological difference between normal and tumour tissue. Some of these genes, e.g. Muc-1, keratin 18 and CD9 are over-expressed in many other cancers.

Gene	Study	Validation
CD24	Welsh *et al.*	RT-PCR
HE4	Welsh *et al.*, Schummer *et al.*	RT-PCR
CD9	Welsh *et al.*	RT-PCR
LU	Welsh *et al.*	RT-PCR
MUC-1	Welsh *et al.*, Schummer *et al.*	RT-PCR
Keratin-18	Welsh *et al.*	RT-PCR
Keratin-8	Welsh *et al.*	RT-PCR
PRAME	Welsh *et al.*,	RT-PCR
ERBB3	Welsh *et al.*	RT-PCR
MEIS 1	Welsh *et al.*	RT-PCR
Paired box gene 8 (PAX8)	Welsh *et al.*	RT-PCR
TACSTD1	Welsh *et al.*	RT-PCR
DDR1	Welsh *et al.*	RT-PCR
OSF2	Matei *et al.*, Ismail *et al.*	RT-PCR, Northern blot
PAI-2	Matei *et al.*	RT-PCR
Integrin, α subunit	Matei *et al.*	RT-PCR
Proto-oncogene (Wnt-5a)	Matei *et al.*	RT-PCR
Frizzled-7	Matei *et al.*	RT-PCR
SPARC/osteonectin	Ismail *et al.*	Northern blot
Prostasin	Mok *et al.*	RT-PCR
Ryudocan	Schummer *et al.*	RT-PCR
Beta actin	Schummer *et al.*	RT-PCR

Table 4.1. Selected genes more than 2-fold over-expressed in my study and others (Welsh *et al,* Schummer *et al.*, Matei *et al.*, Ismail *et al.* and Mok *et al.*) in primary ovarian cancer compared to normal ovarian tissue.

4.2 GEM Profile of Primary and Secondary Ovarian Serous Adenocarcinoma

My study found that the GEM profile of normal ovarian tissue was markedly different from that of the cancers studied. It was expected that the primary tumour would contain a gene expression profile consistent with growth and differentiation, and the secondary deposits to contain genes expressed consistent with metastasis. The gene signature of primary tissue was, however, very similar to that of the secondary spread. This is contrary to the classic model of carcinogenesis. If the metastatic signature is already present in primary cancers, this implies they have the ability to metastasise from the outset. This is now becoming the prevailing view of carcinogenesis. Bernards and Weinberg suggest that a few cells in the initial tumour mass have the molecular signature which confer the ability both for growth and, later, metastasis [Bernards and Weinberg, 2002]. Figure 4.1 demonstrates the two models of metastasis; the emerging view is of a genetic alteration occurring early in carcinogenesis which confers the ability to the tumour to metastasise even at an early stage, so both primary and metastatic tumours have the same GEM profile. The traditional view is that the tumour grows and only later when the tumour is established are the genes necessary for metastasis expressed, so the primary and secondary tumours have different GEM profiles. There are three consequences of this theory: firstly, the mutations necessary for metastasis are already present in the early stages of tumorigenesis; secondly, metastasis-specific genes do not exist; and thirdly, because the metastatic ability is present at the outset, even small tumours can metastasise early. Evidence supporting this view is accumulating:

1. The first comes from the fact that a major risk factor for developing recurrence with solid tumours is the presence of lymph node metastases. Antibodies against epithelial differentiation antigens such as cytokeratins have been developed to detect micrometastases in lymph nodes. Studies have shown that apparently localised (Stage 1) tumours which are found to have occult spread have a worse prognosis; in breast cancer [McGuckin et al., 1996], prostate cancer [Freeman et al., 1995], colon cancer [Greenson et al., 1994] and melanoma [Carlson et al., 2003].

2. Microarray data on breast cancer has been used to subdivide locally advanced tumours into 2 groups, according to their gene profiles, and correlate this to the clinical outcome [Sorlie et al., 2001]. The basal-like and ERBB2+ subtypes had the worst prognoses. This study has demonstrated that the GEM profiles of Stage 1 tumours can be used to predict which tumours will metastasise and that the metastatic signature is already present when the cancer is locally confined. This could have implications for treatment of early stage disease.

3. Further evidence comes from studies showing that metastatic signatures within primary tumours can predict subsequent metastasis. Ramaswamy et al [Ramaswamy et al., 2003] examined the GEM profiles of primary adenocarcinomas of multiple tumour types and compared these to unmatched adenocarcinoma metastases in order to identify differences in GEM profiles between primary tumours and metastases. A distinct profile for each was established, although the GEM profile of metastases was also found in a subset of primary tumours. These tumours were associated with a significantly worse prognosis. Seventeen critical genes were identified to be predictive of spread, some up-regulated, others down-regulated. These same genes were present irrespective of the tumour type, supporting the notion that adenocarcinomas from different sites have similar pathways to metastasis.

My study has found that within the stage III primary serous ovarian adenocarcinomas a number of metastatic predictive genes including EZH2 [Varambally et al., 2002], PTTN and Lamin-B [Ramaswamy et al., 2003] are over-expressed in primary tissue at least as highly as in omental metastases (Figure 3.15). This supports the notion that most tumour cells in advanced primary ovarian lesions have acquired the genetic signature enabling invasion and metastasis. A GEM study comparing stage Ia with stages II, III and IV might identify genes that infer the propensity of ovarian tumour cells to metastasise, although it would be challenging to obtain sufficient material.

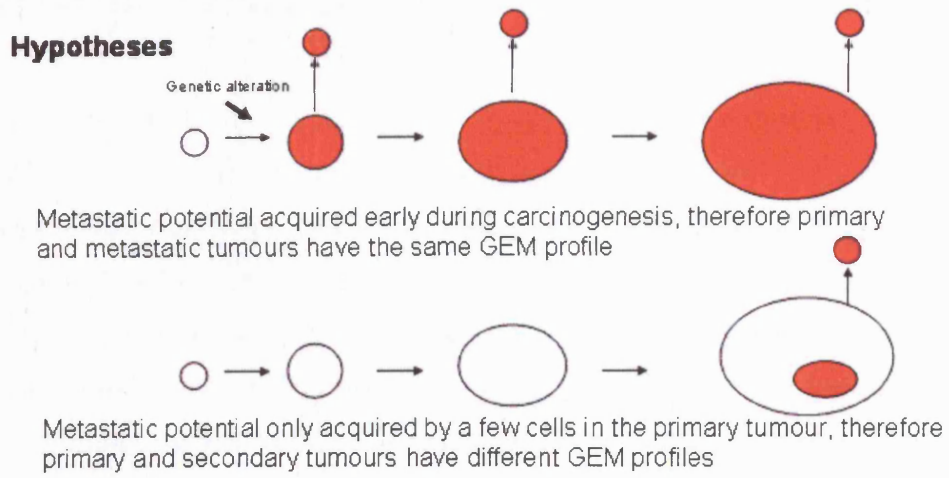


Figure 4.1. Models of metastasis.
The first hypothesis implies a genetic event causing an alteration in the GEM profile of an early cancer which confers the ability to metastasise. Therefore both the primary and secondary tumours have the same GEM profile. This is the hypothesis confirmed by my study, as both the primary tumours and omental lesions have very similar profiles. The classic view implies the metastatic signature is acquired later in the tumour's evolution. This view is now being questioned.

A great number of genes over-expressed in primary tumours were associated with epithelia. This might reflect the epithelial origin of these tumours or a transformed phenotype. HPN for example was marginally over-expressed in both primary and secondary ovarian cancer tissue compared to normal ovary (approximately 2-fold). HPN is a serine protease which has been shown to be over-expressed in prostate cancer cells, and significantly correlates with poor clinical outcome [Dhanasekaran et al., 2001]. Hepsin was further investigated by performing immunohistochemistry; the staining was found to be localised to the epithelial cells, suggesting it may be a marker of epithelia rather than of malignancy (Figure 3.24). However there was a notable difference in the pattern with malignant cells showing a distinct membranous staining, suggestive of heightened secretion.

4.3 Metastatic Spread

Once cancer cells have successfully evaded cell death in the circulation e.g. anoikis, cells lodge in certain organs and extravasate in a very specific manner, depending on the tumour type. In 1889, Paget proposed the "seed and soil" theory where the "seed", the tumour cell, metastasised to a specific organ, the "soil" [Paget, 1889]. It has since then become clear that the sites of metastasis are governed not purely by the neoplastic cells, but also by the microenvironment of the target organ. The exact mechanism is still to be fully elucidated. Ovarian cancer metastasises predominantly to the omentum and peritoneal surfaces. One theory purposes the role of chemokines. Chemokines are immune/inflammatory cytokines that mediate chemotaxis of leukocytes involved in autoimmune and inflammatory diseases [Gerard and Rollins, 2001]. The receptors for chemokines are part of the seven-transmembrane G-protein-coupled receptor family (GPCR) and are mainly expressed on immune and inflammatory cells, such as T and B lymphocytes, dendritic cells, and granulocytes, in which ligand-receptor interactions lead to cell migration. Chemokine expression is either induced at sites of inflammation to recruit inflammatory cells that mediate tissue destruction, or is constitutive in lymphoid organs (i.e., lymph nodes and spleen) to orchestrate the response to antigens, including autoantigens that drive autoimmune diseases. The process of leucocyte maturation, subsequent entry into the circulation and eventual homing to specific tissue sites closely resembles that of tumour cell invasion and metastasis to distant organs. Therefore Müller *et al.* hypothesised that tumour cells may utilise the same chemokines to control metastatic organ specificity [Muller *et al.*, 2001]. Their study demonstrated that specific expression of chemokine receptors on breast cancer cells is an essential event that leads to the homing and metastasis of these tumour cells in a chemokine ligand and receptor–dependent, organ-specific manner. Namely, the chemokine receptors CXCR4 and CCR7 are highly expressed on breast cancer cells and their corresponding ligands, CXCL12/SDF-1a and CCL21/6Ckine are present in the organs to which the breast cancer cells metastasise to (Figure 4.2). This was verified by *in vitro* work, where the CXCL12 ligand induced breast cancer cells to undergo the process of invasion [Muller *et al.*, 2001], including pseudopodial protrusion, directed migration and penetration of ECM barriers. *In vivo* work on mice confirmed these findings, where metastasis to lung tissue containing CXCL12 was blocked by a

neutralising CXCR4 monoclonal antibody.

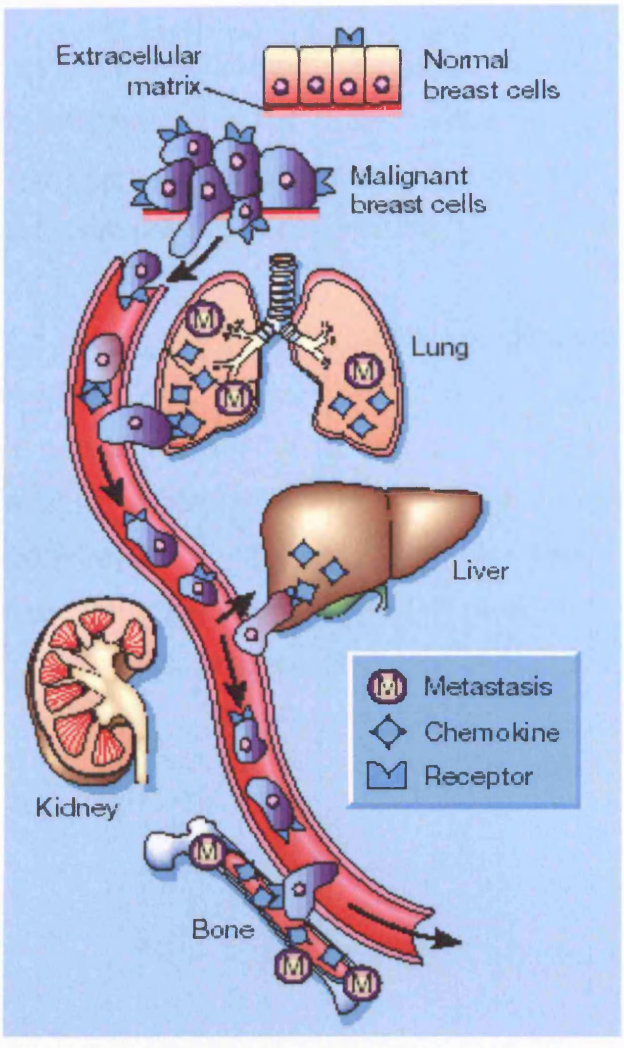


Figure 4.2. Mechanism of chemokine-mediated metastasis of breast cancer.
Figure taken from Muller *et al.*, (2001).

Scotton *et al* researched a panel of chemokines in primary ovarian cancers, and ascites samples, and found that only CXCR4 was reliably expressed on cancer cells [Scotton *et al.*, 2001]. Other chemokines, such as CCR1 were also expressed although only on leucocytes. The ligand for CXCR4, CXCL12, was found in ascites from 63 patients. An *in vitro* migration assay demonstrated ovarian cancer cell movement towards CXCL12. They therefore suggested that a chemokine gradient exists which enables ovarian cancer cells to migrate from the primary tumour mass into the peritoneum. In addition, when ovarian cancer cell lines were treated with CXCL12, β_1 integrin expression on the cell surface was greatly increased, affecting

peritoneal adhesion of cells. This could be a mechanism by which ovarian cancer cells spread to the peritoneal cavity.

My study has shown very similar results (see section 3.8) in that CXCR4 was the most highly expressed chemokine in ovarian cancers. Figures 3.25 and 3.26 show that chemokine expression levels by both semi-quantitative qRT-PCR and microarray analysis are highly concordant.

Chemokines also regulate angiogenesis, both positively and negatively. Angiogenesis is essential for growth, survival, invasion and metastasis of tumours. There is also evidence that chemokines activate tumour cells to secrete enzymes such as MMPs and serine proteases in order to allow migration through the ECM, penetration of the basement membrane and entry into the circulation. CXCL8 induces expression of MMP-2 and MMP-9 in malignant melanoma and prostate cancer cells, respectively [Luca *et al.*, 1997;Inoue *et al.*, 2000].

4.4 Origin of Epithelial Ovarian Cancers

A number of groups have previously investigated gene expression profiling of ovarian cancer using microarrays [Wang et al., 1999;Welsh et al., 2001;Ono et al., 2000;Ismail et al., 2000;Mok et al., 2001b;Shridhar et al., 2001]. These studies have focussed on either the identification of gene products which can serve as ovarian cancer specific markers [Mok et al., 2001b] or on the initiation and progression of ovarian cancer [Ismail et al., 2000;Shridhar et al., 2001]. This has been achieved by comparing normal ovarian epithelium with ovarian cancer samples, as the majority of ovarian cancers are thought to arise from the ovarian surface epithelium [Feeley and Wells, 2001] which exists as a single layer of cells covering the ovaries. The surface epithelium represents a minute cellular component of the ovary, compared to the numerous stromal and germ cells. The epithelial layer easily sloughs off at the time of surgery by manual handling, and it is a challenge to obtain enough cells for use in any experimental procedures. Researchers have overcome this problem by firstly using short-term cell culture to increase the number of cells available [Ismail et al., 2000], secondly by RNA amplification [Ono et al., 2000] and thirdly by using commercially available RNA [Welsh et al., 2001]. These approaches however have drawbacks ; (i) short-term culture favouring the growth of only a subset of epithelial cells, (ii) RNA amplification leading to unequal amplification of all RNA transcripts in the cell population and (iii) the inclusion of a stromal component in commercially available RNA. Table 4.2 summarises ovarian cancer GEM profiling studies comparing ovarian cancer tissue with normal ovaries, the type of array used and the normal ovarian baseline used. Table 4.3 summarises those studies using ovarian cancer cell lines.

Study	Array type	Number of genes	Cancer tissue histology	Normal tissue baseline
Welsh *et al*	Oligonucleotide (glass)	6,800	Serous adenocarcinomas	Normal ovary, macro OSE enrichment
Schummer *et al*	cDNA microarray (nylon filter)	21,500	Mostly serous adenocarcinomas	OSE cells, normal ovarian tissue (mostly stroma), fetal ovary pool
Ono *et al*	cDNA microarray	9,121	Serous and mucinous	Adjacent normal tissue from the same patients
Wang *et al*	cDNA microarray (nylon filter)	5,766	Serous, mucinous, clear cell, endometrioid	Commercial ovarian RNA (nonenriched)
Shridhar *et al*	cDNA microarray (nylon filter)	25,000	Serous, clear cell, endometrioid (high grade, variable stage)	OSE brushings

Table 4.2 GEM profiling studies using cancer cell tissue.

Study	Array type	Number of genes	Cancer cells	Normal cells
Wong *et al*	cDNA microarray (glass)	2,400	In-house developed cell lines; SKOV-3	OSE short-term culture
Ismail *et al*	Subtractive hybridisation/ cDNA microarray	255	In-house cancer cell culture	OSE short-term culture
Tonin *et al*	Oligonucleotide array (glass)	6,416	In-house developed cell lines	OSE short-term culture

Table 4.3 GEM profiling studies using cancer cell lines.

In my study, ovarian epithelium was macrodissected from normal ovarian tissue. Matched primary and secondary metastatic serous ovarian adenocarcinoma specimens were verified histopathologically in five cases to comprise at least 70% tumour. A number of ovarian cancer genes previously identified by GEM were confirmed in this study e.g. CD24 [Welsh et al., 2001], HE4 [Schummer et al., 1999], PRAME [Ismail et al., 2000], B-factor (properdin) [Shridhar et al., 2001], and where the studies overlap, the data are highly consistent despite the difference in methodology.

Tumours rely very heavily on the surrounding stromal cells to participate in the production of growth factors and other products necessary for the cancer to become established (see section 1.5). Global analysis of tumour specimens will give information on the gene expression of all transcripts, but cannot differentiate which

cell is contributing each transcript. Laser capture microdissection (LCM) [Simone et al., 1998] has been developed to obtain pure populations of cells from heterogeneous tissue. Individual cells can be selected and "captured" from cell aggregates or tissue. Frozen, formalin-fixed or paraffin-embedded tissue sections are processed, stained, and dehydrated, then scanned under an inverted microscope. Cells are visualized through a thermoplastic film which is attached to the bottom of an optically clear microfuge tube cap. A laser pulse, directed onto the cells of interest, melts the film and allows it to flow onto the targeted area where it cools and bonds with the underlying cell(s). The film along with the adhered cells or clusters is then lifted. Captured cells can then be used for microarray studies (see Figure 4.3).

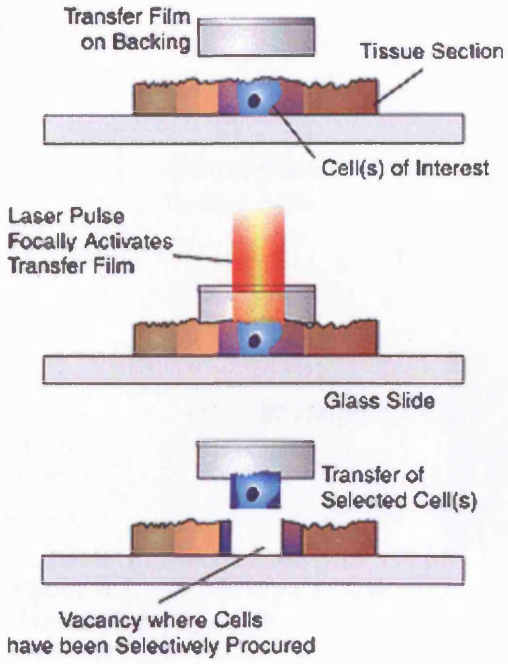


Figure 4.3. Laser capture microdissection.
A special thermoplastic film is coated onto to a small plastic cap that fits into a microfuge tube. The cap is then placed on the tissue to be microdissected, which becomes a part of the focal length of the microscope. When the targeted cells are identified, a low-power infrared laser pulse melts and anneals the film to the tissue. The cap is lifted and the tissue is captured for further analysis. An archival workstation allows photographic documentation of every step. This system ensures that biological molecules such as RNA and DNA, remain undamaged during the microdissection process. Figure taken from http://www.arctur.com.

LCM is usually carried out on frozen sections when samples will be used for RNA isolation. This is because formalin fixation introduces crosslinks between nucleic acids and proteins, resulting in fragmentation of the RNA. Freezing avoids crosslinking but introduces a different problem. Endogenous nucleases are not irreversibly inactivated, and can become active during the tissue processing steps (fixation, staining, destaining, and dehydration) carried out after sectioning. Figure 4.4 shows a summary of the microdissection process.

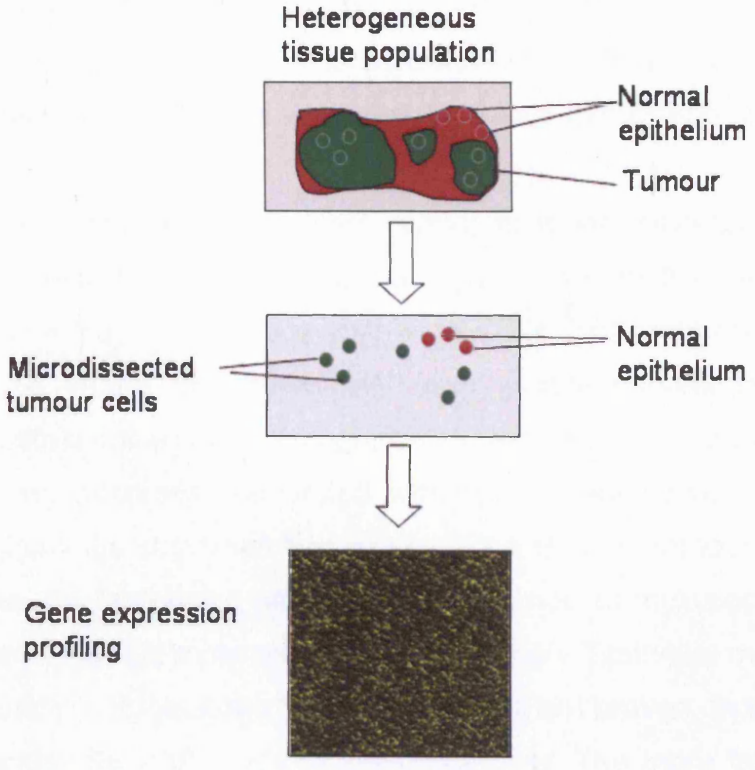


Figure 4.4. LCM on a heterogeneous tissue sample.
The heterogeneous tumour tissue is stained in order to identify the cancer cell subpopulation. LCM then captures normal cells and cancer cells separately, and these can then be processed for GEM analysis.

Analysis of cancer tissue by global survey and microdissection both have advantages and disadvantages. An advantage of the global survey approach include the use of a higher amount of starting material, and sufficient RNA can be extracted for microarray target preparation without the need for amplification. This method is also much less labour intensive. The disadvantage is that the actual percentage of the cancer cell population under study is unknown; if the percentage is low, then the signal from the cancer cell population may be obscured by the higher-abundance

normal cell subpopulation. The advantage of microdissection is that clearly defined subpopulations can be directly compared. However, the disadvantages are due to the high level of expertise and resources that are required. Specialised training is required to use the LCM apparatus, and a trained pathologist must identify the cells to be extracted. Even though pure cell populations are obtained, only single cells are extracted so RNA invariably must be amplified. This technique is excellent for separating out normal, carcinoma in situ and invasive cancer cells from the same tissue. Sgroi et al [Sgroi et al., 1999] used LCM to isolate normal and invasive metastatic breast cancer cells from a single patient, and compared their gene expression profiles. Kitahara et al [Kitahara et al., 2001] also isolated normal and colorectal tumour populations and compared their expression profiles.

Applying this technique to ovarian cancer is more challenging. Although ovarian cancer can arise from any of the cell types found in the ovary, around 90% are derived from the ovarian surface epithelium. This epithelium covers the entire ovarian surface, and varies morphologically from simple squamous to cuboidal to low pseudostratified columnar. Although the ovarian surface is generally smooth early in life, the ovary becomes convoluted with age. Invaginations of the epithelium form crypts or gland-like structures that can become pinched off to form epithelial inclusion cysts within the underlying stroma. The incidence of inclusion cysts increases with age and are common in postmenopausal women. Epithelial ovarian cancers have a similar incidence. It has been hypothesised, but not proven, that these inclusion cysts are a potential origin of many epithelial cancers. The more frequent appearance of epithelial invaginations and inclusion cysts in women with hereditary risk of ovarian cancer has strengthened this hypothesis [Salazar et al., 1996]. In addition, some microscopic borderline and malignant tumours have been observed to arise directly within these sites, and they are often associated with dysplasia in inclusion cysts in the same or contralateral ovary [Deligdisch and Gil, 1989;Scully, 1995]. LCM studies would be invaluable in identifying GEM alterations in the cells from these structures.

4.5 Ovarian Cancer Biomarkers

The early detection of ovarian cancer is vital for its ultimate control and prevention. The effectiveness of conventional therapeutics is limited once metastasis has occurred. Late-stage diagnosis of ovarian cancer can be attributed to the fact that the disease is relatively "asymptomatic" in its early stages, and that the symptoms of late stage disease, such as abdominal discomfort, weight loss, diarrhoea or constipation, vaginal bleeding and shortness of breath, are non-specific complaints. Consequently the overall 5 year survival is around 25%. To be effective, a clinically useful biomarker should be detectable in accessible body fluids such as blood, urine or saliva.

Molecular biomarkers are essential tools for detecting and monitoring cancer. The marker most extensively used in ovarian cancer is CA125, a high molecular weight glycoprotein which is expressed by tissues derived from the coelomic epithelium. The secreted levels are proportional to the tumour volume; hence only 50% of stage I tumours have elevated levels of CA125, which reduces its effectiveness as a screening marker [Jacobs and Bast, Jr., 1989]. An algorithm has been calculated [Skates et al., 1995] which takes into account sequential changes in CA125 levels over time, rather than a single reading. Other markers for ovarian cancer detection have been investigated, including Lysophosphatidic acid (LPA) [Xu et al., 1998], macrophage colony-stimulating factor (MCS-F) [Suzuki et al., 1995], ovarian carcinoma-associated antigen (OCA) [Knauf and Urbach, 1978] and tumour-associated trypsin inhibitor (TATI) [Medl et al., 1995]. However, none of these markers has proved superior to CA125.

This study has identified a potential new biomarker MGB2 which is significantly over-expressed in primary and metastatic ovarian cancer compared to normal ovarian tissue. MGB2 is also increased in LMP and will therefore not be useful on its own as a tumour marker, but might still be helpful in combination with other markers. MGB2 has not previously been described in ovarian cancer. This gene, located on chromosome 11q13 is part of the uteroglobin family and was first sequenced by a group screening a human genomic library [Becker et al., 1998]. It has high sequence homology to mammaglobin 1 (MGB1) which is present in normal breast epithelia and

frequently up-regulated in breast cancers [Watson and Fleming, 1996;Watson et al., 1998]. MGB2 has been detected in axillary lymph node micrometastases of breast cancer patients [Ooka et al., 2000] and is over-expressed in endometrioid endometrial carcinomas compared to nonendometrioid cancers by cDNA microarray analysis [Moreno-Bueno et al., 2003]. Both endometrioid and breast cancers are oestrogen-dependent, and may explain a mechanism for the over-expression of MGB2. The link between ovarian cancer and oestrogen is less clear. The women's health initiative (WHI) trial [Anderson et al., 2003;Ooka et al., 2000] reported a non-significant excess of ovarian cancer in women taking combined oestrogen and progesterone hormone replacement therapy compared to non-users. The belief is generally not held that ovarian cancer is directly influenced by oestrogen, indicating that MGB2 may be operating in a different manner in these cancers.

The qRT-PCR analysis of MGB2 confirmed the over-expression of MGB2 in primary and metastatic ovarian cancer in this study and showed that it also demonstrated high expression in LMP samples. LMP tumours are a distinct subtype of ovarian neoplasm which generally run a benign course with only 0.7% conversion to invasive disease. No biomarker to date is sufficiently specific for screening and monitoring disease progression in LMP tumours. Further studies are needed to test MGB2 as a biomarker for this cancer subgroup.

The only widely used ovarian cancer marker CA125 lacks specificity (CA125 or MUC16 is not present on the U95Av2 array). Within the panel of data available on other cancers, MGB2 appears to be a specific biomarker for ovarian tumours with low expression in most normal epithelial tissues and prostate and lung tumours. This survey was far from exhaustive relying on available published GEM data. The screening and selection of other candidates for serological study will benefit from more publicly available data, in particular, data on breast cancer. A combination of MGB2 and other biomarkers may give a more specific signature for epithelial ovarian carcinoma. This study demonstrates that GEM studies are a practical and economical prelude to streamline candidate genes for larger serological studies.

4.6 The Future

I have investigated gene expression differences between normal ovary, primary and corresponding secondary omental serous adenocarcinoma in six patients. Although my findings have been replicated by other authors, ideally I would like to use more samples to make the work more substantive. Using more samples would enable me to correlate outcome with specific gene expression profiles. However, clearly, oligonucleotide arrays and the array experiments are costly which is why my study was limited. Any possible future work I describe here is meant if finances were not an issue.

Another way to take this work forwards is to compare the paired samples O and M used in this thesis to identify whether similarities exist between tumours which would contribute to the understanding of the pathogenesis of serous ovarian adenocarcinoma.
MGB2 is raised in both invasive cancers but also in LMP tumours. Investigating a larger series of both LMP and invasive cancers may identify whether MGB2 is raised in all LMP tumours or only in the small subset which become invasive.

Finally, I would like to test a panel of markers on serum samples from patients with ovarian cancer, benign gynaecological conditions, and other cancers and identify whether it is possible to define a fingerprint for serous ovarian cancer.

CONCLUSION

Ovarian cancer has the highest mortality rate of all the gynaecological malignancies. The lack of specific signs and symptoms of early disease means more than 70% of women present at a late stage, where cytoreductive surgery and conventional chemotherapies have limited effect. Around 60% of women die of their disease. The current marker CA125 is not sufficiently sensitive to use as a mass screening tool in the general population, even when coupled with ultrasound scanning. There is an urgent need to discover a new strategy for ovarian cancer screening.

This study has used high density oligonucleotide arrays to compare the gene expression profiles of normal ovarian epithelium with primary serous adenocarcinoma and serous omental metastases from the same patients. The aims were to firstly identify genes involved in ovarian cancer progression and secondly identify new biomarkers which may be used as screening tools.

The main findings were the GEM profiles of primary and secondary cancers were more similar than dissimilar. Unlike Vogelstein's model for colon cancer, no definite progression pathway was identified for ovarian cancer. This may imply that ovarian tumours, at an early stage, have the metastatic profile which will predestine them to metastasise even when the tumour is localised. There was a definite difference in GEM profiles between normal and cancer samples. The other main finding was one gene, not yet described in epithelial ovarian cancer, mammaglobin 2 (MGB2) was significantly over-expressed in primary and secondary disease compared to normal ovary. This gene meets the criteria for biomarkers, that is, it is secreted and is not expressed in other normal or cancer tissues at significant levels. Serum studies would show whether this marker has clinical usefulness, either alone, or as part of a panel of biomarkers.

REFERENCES

't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M, Peterse HL, van der KK, Marton MJ, Witteveen AT, Schreiber GJ, Kerkhoven RM, Roberts C, Linsley PS, Bernards R, Friend SH (2002) Gene expression profiling predicts clinical outcome of breast cancer. *Nature* **415**: 530-536

Aarnio M, Mecklin JP, Aaltonen LA, Nystrom-Lahti M, Jarvinen HJ (1995) Life-time risk of different cancers in hereditary non-polyposis colorectal cancer (HNPCC) syndrome. *Int J Cancer* **64**: 430-433

Achen MG, Jeltsch M, Kukk E, Makinen T, Vitali A, Wilks AF, Alitalo K, Stacker SA (1998) Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). *Proc Natl Acad Sci U S A* **95**: 548-553

Adams MD, Kelley JM, Gocayne JD, Dubnick M, Polymeropoulos MH, Xiao H, Merril CR, Wu A, Olde B, Moreno RF, . (1991) Complementary DNA sequencing: expressed sequence tags and human genome project. *Science* **252**: 1651-1656

Afzal S, Lalani e, Foulkes WD, Boyce B, Tickle S, Cardillo MR, Baker T, Pignatelli M, Stamp GW (1996) Matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-2 expression and synthetic matrix metalloproteinase-2 inhibitor binding in ovarian carcinomas and tumor cell lines. *Lab Invest* **74**: 406-421

Agrawal D, Chen T, Irby R, Quackenbush J, Chambers AF, Szabo M, Cantor A, Coppola D, Yeatman TJ (2002) Osteopontin identified as lead marker of colon cancer progression, using pooled sample expression profiling. *J Natl Cancer Inst* **94**: 513-521

Alizadeh AA, Eisen MB, Davis RE, Ma C, Lossos IS, Rosenwald A, Boldrick JC, Sabet H, Tran T, Yu X, Powell JI, Yang L, Marti GE, Moore T, Hudson J, Jr., Lu L, Lewis DB, Tibshirani R, Sherlock G, Chan WC, Greiner TC, Weisenburger DD, Armitage JO, Warnke R, Levy R, Wilson W, Grever MR, Byrd JC, Botstein D, Brown PO, Staudt LM (2000) Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature* **403**: 503-511

Alwine JC, Kemp DJ, Stark GR (1977) Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. *Proc Natl Acad Sci U S A* **74**: 5350-5354

Anderson GL, Judd HL, Kaunitz AM, Barad DH, Beresford SA, Pettinger M, Liu J, McNeeley SG, Lopez AM (2003) Effects of estrogen plus progestin on gynecologic cancers and associated diagnostic procedures: the Women's Health Initiative randomized trial. *JAMA* **290**: 1739-1748

Anttila M, Kosma VM, Ji H, Wei-Ling X, Puolakka J, Juhola M, Saarikoski S, Syrjanen K (1998) Clinical significance of alpha-catenin, collagen IV, and Ki-67 expression in epithelial ovarian cancer. *J Clin Oncol* **16**: 2591-2600

Baker VV, Borst MP, Dixon D, Hatch KD, Shingleton HM, Miller D (1990) c-myc

amplification in ovarian cancer. *Gynecol Oncol* **38**: 340-342

Barreca A, Minuto F (1989) Somatomedins: chemical and functional characteristics of the different molecular forms. *J Endocrinol Invest* **12**: 279-293

Bartlett JM, Rabiasz GJ, Scott WN, Langdon SP, Smyth JF, Miller WR (1992) Transforming growth factor-beta mRNA expression and growth control of human ovarian carcinoma cells. *Br J Cancer* **65**: 655-660

Bast RC, Jr., Klug TL, St John E, Jenison E, Niloff JM, Lazarus H, Berkowitz RS, Leavitt T, Griffiths CT, Parker L, Zurawski VR, Jr., Knapp RC (1983) A radioimmunoassay using a monoclonal antibody to monitor the course of epithelial ovarian cancer. *N Engl J Med* **309**: 883-887

Battaglia F, Scambia G, Benedetti PP, Baiocchi G, Perrone L, Iacobelli S, Mancuso S (1989) Epidermal growth factor receptor expression in gynecological malignancies. *Gynecol Obstet Invest* **27**: 42-44

Bauknecht T, Angel P, Kohler M, Kommoss F, Birmelin G, Pfleiderer A, Wagner E (1993) Gene structure and expression analysis of the epidermal growth factor receptor, transforming growth factor-alpha, myc, jun, and metallothionein in human ovarian carcinomas. Classification of malignant phenotypes. *Cancer* **71**: 419-429

Bauknecht T, Runge M, Schwall M, Pfleiderer A (1988) Occurrence of epidermal growth factor receptors in human adnexal tumors and their prognostic value in advanced ovarian carcinomas. *Gynecol Oncol* **29**: 147-157

Beck EP, Russo P, Gliozzo B, Jaeger W, Papa V, Wildt L, Pezzino V, Lang N (1994) Identification of insulin and insulin-like growth factor I (IGF I) receptors in ovarian cancer tissue. *Gynecol Oncol* **53**: 196-201

Becker RM, Darrow C, Zimonjic DB, Popescu NC, Watson MA, Fleming TP (1998) Identification of mammaglobin B, a novel member of the uteroglobin gene family. *Genomics* **54**: 70-78

Beckmann MW, Niederacher D, Schnurch HG, Gusterson BA, Bender HG (1997) Multistep carcinogenesis of breast cancer and tumour heterogeneity. *J Mol Med* **75**: 429-439

Bellacosa A, de Feo D, Godwin AK, Bell DW, Cheng JQ, Altomare DA, Wan M, Dubeau L, Scambia G, Masciullo V, . (1995) Molecular alterations of the AKT2 oncogene in ovarian and breast carcinomas. *Int J Cancer* **64**: 280-285

Berchuck A (1995) Biomarkers in the ovary. *J Cell Biochem Suppl* **23**: 223-226

Berchuck A, Heron KA, Carney ME, Lancaster JM, Fraser EG, Vinson VL, Deffenbaugh AM, Miron A, Marks JR, Futreal PA, Frank TS (1998) Frequency of germline and somatic BRCA1 mutations in ovarian cancer. *Clin Cancer Res* **4**: 2433-2437

Berchuck A, Iversen ES, Lancaster JM, Pittman J, Luo J, Lee P, Murphy S, Dressman HK, Febbo PG, West M, Nevins JR, Marks JR (2005) Patterns of gene expression that characterize long-term survival in advanced stage serous ovarian

cancers. *Clin Cancer Res* **11**: 3686-3696

Berchuck A, Kohler MF, Marks JR, Wiseman R, Boyd J, Bast RC, Jr. (1994) The p53 tumor suppressor gene frequently is altered in gynecologic cancers. *Am J Obstet Gynecol* **170**: 246-252

Berchuck A, Olt GJ, Everitt L, Soisson AP, Bast RC, Jr., Boyer CM (1990) The role of peptide growth factors in epithelial ovarian cancer. *Obstet Gynecol* **75**: 255-262

Berchuck A, Rodriguez GC, Kamel A, Dodge RK, Soper JT, Clarke-Pearson DL, Bast RC, Jr. (1991) Epidermal growth factor receptor expression in normal ovarian epithelium and ovarian cancer. I. Correlation of receptor expression with prognostic factors in patients with ovarian cancer. *Am J Obstet Gynecol* **164**: 669-674

Berk AJ, Sharp PA (1977) Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. *Cell* **12**: 721-732

Bernards R, Weinberg RA (2002) Metastasis genes: A progression puzzle. *Nature* **418**: 823

Bhattacharjee A, Richards WG, Staunton J, Li C, Monti S, Vasa P, Ladd C, Beheshti J, Bueno R, Gillette M, Loda M, Weber G, Mark EJ, Lander ES, Wong W, Johnson BE, Golub TR, Sugarbaker DJ, Meyerson M (2001) Classification of human lung carcinomas by mRNA expression profiling reveals distinct adenocarcinoma subclasses. *Proc Natl Acad Sci U S A* **98**: 13790-13795

Bicher A, Ault K, Kimmelman A, Gershenson D, Reed E, Liang B (1997) Loss of heterozygosity in human ovarian cancer on chromosome 19q. *Gynecol Oncol* **66**: 36-40

Blaakaer J, Micic S, Morris ID, Hording U, Bennett P, Toftager-Larsen K, Djursing H, Bock JE (1993) Immunoreactive inhibin-production in post-menopausal women with malignant epithelial ovarian tumors. *Eur J Obstet Gynecol Reprod Biol* **52**: 105-110

Brown E, Stewart M, Rye T, Al-Nafussi A, Williams AR, Bradburn M, Smyth J, Gabra H (2004) Carcinosarcoma of the ovary: 19 years of prospective data from a single center. *Cancer* **100**: 2148-2153

Brown MP, Grundy WN, Lin D, Cristianini N, Sugnet CW, Furey TS, Ares M, Jr., Haussler D (2000) Knowledge-based analysis of microarray gene expression data by using support vector machines. *Proc Natl Acad Sci U S A* **97**: 262-267

Brown TJ, Shaw PA, Karp X, Huynh MH, Begley H, Ringuette MJ (1999) Activation of SPARC expression in reactive stroma associated with human epithelial ovarian cancer. *Gynecol Oncol* **75**: 25-33

Burghardt E, Girardi F, Lahousen M, Tamussino K, Stettner H (1991) Patterns of pelvic and paraaortic lymph node involvement in ovarian cancer. *Gynecol Oncol* **40**: 103-106

But I, Gorisek B (2000) DNA-ploidy as an independent prognostic factor in patients with serous ovarian carcinoma. *Int J Gynaecol Obstet* **71**: 259-262

Cancer Research UK. Scientific Yearbook 2001-2. 2002.
Ref Type: Generic

Carlson GW, Murray DR, Lyles RH, Staley CA, Hestley A, Cohen C (2003) The amount of metastatic melanoma in a sentinel lymph node: does it have prognostic significance? *Ann Surg Oncol* **10**: 575-581

Carraway KL, III, Cantley LC (1994) A neu acquaintance for erbB3 and erbB4: a role for receptor heterodimerization in growth signaling. *Cell* **78**: 5-8

Casey G, Lopez ME, Ramos JC, Plummer SJ, Arboleda MJ, Shaughnessy M, Karlan B, Slamon DJ (1996) DNA sequence analysis of exons 2 through 11 and immunohistochemical staining are required to detect all known p53 alterations in human malignancies. *Oncogene* **13**: 1971-1981

Chambers SK, Gertz RE, Jr., Ivins CM, Kacinski BM (1995) The significance of urokinase- type plasminogen activator, its inhibitors, and its receptor in ascites of patients with epithelial ovarian cancer. *Cancer* **75**: 1627-1633

Chang BD, Swift ME, Shen M, Fang J, Broude EV, Roninson IB (2002) Molecular determinants of terminal growth arrest induced in tumor cells by a chemotherapeutic agent. *Proc Natl Acad Sci U S A* **99**: 389-394

Chenevix-Trench G, Leary J, Kerr J, Michel J, Kefford R, Hurst T, Parsons PG, Friedlander M, Khoo SK (1992) Frequent loss of heterozygosity on chromosome 18 in ovarian adenocarcinoma which does not always include the DCC locus. *Oncogene* **7**: 1059-1065

Cheng JQ, Godwin AK, Bellacosa A, Taguchi T, Franke TF, Hamilton TC, Tsichlis PN, Testa JR (1992) AKT2, a putative oncogene encoding a member of a subfamily of protein-serine/threonine kinases, is amplified in human ovarian carcinomas. *Proc Natl Acad Sci U S A* **89**: 9267-9271

Cheng P, Schmutte C, Cofer KF, Felix JC, Yu MC, Dubeau L (1997) Alterations in DNA methylation are early, but not initial, events in ovarian tumorigenesis. *Br J Cancer* **75**: 396-402

Chien CH, Huang CC, Lin YH, Shen J, Chow SN (1997) Detection of serum transforming growth factor-alpha in patients of primary epithelial ovarian cancers by enzyme immunoassay. *Gynecol Oncol* **66**: 405-410

Choi C, Cho S, Horikawa I, Berchuck A, Wang N, Cedrone E, Jhung SW, Lee JB, Kerr J, Chenevix-Trench G, Kim S, Barrett JC, Koi M (1997) Loss of heterozygosity at chromosome segment Xq25-26.1 in advanced human ovarian carcinomas. *Genes Chromosomes Cancer* **20**: 234-242

Cirisano FD, Karlan BY (1996) The role of the HER-2/neu oncogene in gynecologic cancers. *J Soc Gynecol Investig* **3**: 99-105

Clark EA, Golub TR, Lander ES, Hynes RO (2000) Genomic analysis of metastasis reveals an essential role for RhoC. *Nature* **406**: 532-535

Claus EB, Schildkraut JM, Thompson WD, Risch NJ (1996) The genetic attributable

risk of breast and ovarian cancer. *Cancer* **77**: 2318-2324

Colitti CV, Rodabaugh KJ, Welch WR, Berkowitz RS, Mok SC (1998) A novel 4 cM minimal deletion unit on chromosome 6q25.1-q25.2 associated with high grade invasive epithelial ovarian carcinomas. *Oncogene* **16**: 555-559

Cooke I, O'Brien M, Charnock FM, Groome N, Ganesan TS (1995) Inhibin as a marker for ovarian cancer. *Br J Cancer* **71**: 1046-1050

Couch FJ, Farid LM, DeShano ML, Tavtigian SV, Calzone K, Campeau L, Peng Y, Bogden B, Chen Q, Neuhausen S, Shattuck-Eidens D, Godwin AK, Daly M, Radford DM, Sedlacek S, Rommens J, Simard J, Garber J, Merajver S, Weber BL (1996) BRCA2 germline mutations in male breast cancer cases and breast cancer families. *Nat Genet* **13**: 123-125

Cramer DW, Welch WR (1983) Determinants of ovarian cancer risk. II. Inferences regarding pathogenesis. *J Natl Cancer Inst* **71**: 717-721

Cramer DW, Welch WR, Scully RE, Wojciechowski CA (1982) Ovarian cancer and talc: a case-control study. *Cancer* **50**: 372-376

Cramer DW, Xu H (1995) Epidemiologic evidence for uterine growth factors in the pathogenesis of ovarian cancer. *Ann Epidemiol* **5**: 310-314

Crickard K, Gross JL, Crickard U, Yoonessi M, Lele S, Herblin WF, Eidsvoog K (1994) Basic fibroblast growth factor and receptor expression in human ovarian cancer. *Gynecol Oncol* **55**: 277-284

Dang CV, Resar LM, Emison E, Kim S, Li Q, Prescott JE, Wonsey D, Zeller K (1999) Function of the c-Myc oncogenic transcription factor. *Exp Cell Res* **253**: 63-77

Davies BR, Worsley SD, Ponder BA (1998) Expression of E-cadherin, alpha-catenin and beta-catenin in normal ovarian surface epithelium and epithelial ovarian cancers. *Histopathology* **32**: 69-80

De CL, Marchionni L, Gariboldi M, Reid JF, Lagonigro MS, Caramuta S, Ferrario C, Bussani E, Mezzanzanica D, Turatti F, Delia D, Daidone MG, Oggionni M, Bertuletti N, Ditto A, Raspagliesi F, Pilotti S, Pierotti MA, Canevari S, Schneider C (2004) Gene expression profiling of advanced ovarian cancer: characterization of a molecular signature involving fibroblast growth factor 2. *Oncogene* **23**: 8171-8183

Deligdisch L, Gil J (1989) Characterization of ovarian dysplasia by interactive morphometry. *Cancer* **63**: 748-755

Demopoulos RI, Bigelow B, Blaustein A, Chait J, Gutman E, Dubin N (1984) Characterization and survival of patients with serous cystadenocarcinoma of the ovaries. *Obstet Gynecol* **64**: 557-563

Deng Y, Wu X (2000) Peg3/Pw1 promotes p53-mediated apoptosis by inducing Bax translocation from cytosol to mitochondria. *Proc Natl Acad Sci U S A* **97**: 12050-12055

DePriest PD, van NJ, Jr., Gallion HH, Shenson D, Hunter JE, Andrews SJ, Powell

DE, Pavlik EJ (1993) Ovarian cancer screening in asymptomatic postmenopausal women. *Gynecol Oncol* **51**: 205-209

Devlin J, Elder PA, Gabra H, Steel CM, Knowles MA (1996) High frequency of chromosome 9 deletion in ovarian cancer: evidence for three tumour-suppressor loci. *Br J Cancer* **73**: 420-423

Dhanasekaran SM, Barrette TR, Ghosh D, Shah R, Varambally S, Kurachi K, Pienta KJ, Rubin MA, Chinnaiyan AM (2001) Delineation of prognostic biomarkers in prostate cancer. *Nature* **412**: 822-826

Di Cristofano A, Pandolfi PP (2000) The multiple roles of PTEN in tumor suppression. *Cell* **100**: 387-390

Diamandis EP, Yousef GM (2002a) Human tissue kallikreins: a family of new cancer biomarkers. *Clin Chem* **48**: 1198-1205

Diamandis EP, Yousef GM (2002b) Human tissue kallikreins: a family of new cancer biomarkers. *Clin Chem* **48**: 1198-1205

Diebold J, Mosinger K, Peiro G, Pannekamp U, Kaltz C, Baretton GB, Meier W, Lohrs U (2000) 20q13 and cyclin D1 in ovarian carcinomas. Analysis by fluorescence in situ hybridization. *J Pathol* **190**: 564-571

Dimitroulakos J, Marhin WH, Tokunaga J, Irish J, Gullane P, Penn LZ, Kamel-Reid S (2002) Microarray and biochemical analysis of lovastatin-induced apoptosis of squamous cell carcinomas. *Neoplasia* **4**: 337-346

Dodson MK, Cliby WA, Xu HJ, DeLacey KA, Hu SX, Keeney GL, Li J, Podratz KC, Jenkins RB, Benedict WF (1994) Evidence of functional RB protein in epithelial ovarian carcinomas despite loss of heterozygosity at the RB locus. *Cancer Res* **54**: 610-613

Dodson MK, Hartmann LC, Cliby WA, DeLacey KA, Keeney GL, Ritland SR, Su JQ, Podratz KC, Jenkins RB (1993) Comparison of loss of heterozygosity patterns in invasive low-grade and high-grade epithelial ovarian carcinomas. *Cancer Res* **53**: 4456-4460

Donninger H, Bonome T, Radonovich M, Pise-Masison CA, Brady J, Shih JH, Barrett JC, Birrer MJ (2004) Whole genome expression profiling of advance stage papillary serous ovarian cancer reveals activated pathways. *Oncogene* **23**: 8065-8077

Downward J (1998) Ras signalling and apoptosis. *Curr Opin Genet Dev* **8**: 49-54

Doyle P, Maconochie N, Beral V, Swerdlow AJ, Tan SL (2002) Cancer incidence following treatment for infertility at a clinic in the UK. *Hum Reprod* **17**: 2209-2213

Duggan BD, Wan M, Yu MC, Roman LD, Muderspach LI, Delgadillo E, Li WZ, Martin SE, Dubeau L (1998) Detection of ovarian cancer cells: comparison of a telomerase assay and cytologic examination. *J Natl Cancer Inst* **90**: 238-242

Einhorn N, Sjovall K, Knapp RC, Hall P, Scully RE, Bast RC, Jr., Zurawski VR, Jr. (1992) Prospective evaluation of serum CA 125 levels for early detection of ovarian

cancer. *Obstet Gynecol* **80**: 14-18

Eisen MB, Spellman PT, Brown PO, Botstein D (1998) Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci U S A* **95**: 14863-14868

Eissa S, Khalifa A, Laban M, Elian A, Bolton WE (1998) Comparison of flow cytometric DNA content analysis in fresh and paraffin-embedded ovarian neoplasms: a prospective study. *Br J Cancer* **77**: 421-425

Englefield P, Foulkes WD, Campbell IG (1994) Loss of heterozygosity on chromosome 22 in ovarian carcinoma is distal to and is not accompanied by mutations in NF2 at 22q12. *Br J Cancer* **70**: 905-907

Enomoto T, Inoue M, Perantoni AO, Terakawa N, Tanizawa O, Rice JM (1990) K-ras activation in neoplasms of the human female reproductive tract. *Cancer Res* **50**: 6139-6145

Espey LL (1994) Current status of the hypothesis that mammalian ovulation is comparable to an inflammatory reaction. *Biol Reprod* **50**: 233-238

Fathalla MF (1971) Incessant ovulation--a factor in ovarian neoplasia? *Lancet* **2**: 163

Feeley KM, Wells M (2001) Precursor lesions of ovarian epithelial malignancy. *Histopathology* **38**: 87-95

Feldkamper M, Enderle-Schmitt U, Hackenberg R, Schulz KD (1994) Urinary excretion of growth factors in patients with ovarian cancer. *Eur J Cancer* **30A**: 1851-1858

Flyvbjerg A, Mogensen O, Mogensen B, Nielsen OS (1997) Elevated serum insulin-like growth factor-binding protein 2 (IGFBP-2) and decreased IGFBP-3 in epithelial ovarian cancer: correlation with cancer antigen 125 and tumor-associated trypsin inhibitor. *J Clin Endocrinol Metab* **82**: 2308-2313

Folkman J (1971) Tumor angiogenesis: therapeutic implications. *N Engl J Med* **285**: 1182-1186

Ford D, Easton DF, Bishop DT, Narod SA, Goldgar DE (1994) Risks of cancer in BRCA1-mutation carriers. Breast Cancer Linkage Consortium. *Lancet* **343**: 692-695

Ford D, Easton DF, Peto J (1995) Estimates of the gene frequency of BRCA1 and its contribution to breast and ovarian cancer incidence. *Am J Hum Genet* **57**: 1457-1462

Ford D, Easton DF, Stratton M, Narod S, Goldgar D, Devilee P, Bishop DT, Weber B, Lenoir G, Chang-Claude J, Sobol H, Teare MD, Struewing J, Arason A, Scherneck S, Peto J, Rebbeck TR, Tonin P, Neuhausen S, Barkardottir R, Eyfjord J, Lynch H, Ponder BA, Gayther SA, Zelada-Hedman M, . (1998) Genetic heterogeneity and penetrance analysis of the BRCA1 and BRCA2 genes in breast cancer families. The Breast Cancer Linkage Consortium. *Am J Hum Genet* **62**: 676-689

Foulkes WD, Black DM, Stamp GW, Solomon E, Trowsdale J (1993) Very frequent loss of heterozygosity throughout chromosome 17 in sporadic ovarian carcinoma. *Int*

J Cancer **54**: 220-225

Freeman JA, Esrig D, Grossfeld GD, Stein JP, Chen SC, Young LL, Taylor CR, Skinner DG, Lieskovsky G, Cote RJ (1995) Incidence of occult lymph node metastases in pathological stage C (pT3N0) prostate cancer. *J Urol* **154**: 474-478

Frevert EU, Kahn BB (1997) Differential effects of constitutively active phosphatidylinositol 3-kinase on glucose transport, glycogen synthase activity, and DNA synthesis in 3T3-L1 adipocytes. *Mol Cell Biol* **17**: 190-198

Fujioka T, Takebayashi Y, Kihana T, Kusanagi Y, Hamada K, Ochi H, Uchida T, Fukumoto M, Ito M (2001) Expression of E-cadherin and beta-catenin in primary and peritoneal metastatic ovarian carcinoma. *Oncol Rep* **8**: 249-255

Galani E, Sgouros J, Petropoulou C, Janinis J, Aravantinos G, Dionysiou-Asteriou D, Skarlos D, Gonos E (2002) Correlation of MDR-1, nm23-H1 and H Sema E gene expression with histopathological findings and clinical outcome in ovarian and breast cancer patients. *Anticancer Res* **22**: 2275-2280

Gamallo C, Palacios J, Moreno G, Calvo dM, Suarez A, Armas A (1999) beta-catenin expression pattern in stage I and II ovarian carcinomas : relationship with beta-catenin gene mutations, clinicopathological features, and clinical outcome. *Am J Pathol* **155**: 527-536

Garg PP, Kerlikowske K, Subak L, Grady D (1998) Hormone replacement therapy and the risk of epithelial ovarian carcinoma: a meta-analysis. *Obstet Gynecol* **92**: 472-479

Gerard C, Rollins BJ (2001) Chemokines and disease. *Nat Immunol* **2**: 108-115

Gerhold D, Lu M, Xu J, Austin C, Caskey CT, Rushmore T (2001) Monitoring expression of genes involved in drug metabolism and toxicology using DNA microarrays. *Physiol Genomics* **5**: 161-170

Gillett CE, Barnes DM (1998) Demystified ... cell cycle. *Mol Pathol* **51**: 310-316

Golub TR, Slonim DK, Tamayo P, Huard C, Gaasenbeek M, Mesirov JP, Coller H, Loh ML, Downing JR, Caligiuri MA, Bloomfield CD, Lander ES (1999) Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. *Science* **286**: 531-537

Green A, Purdie D, Bain C, Siskind V, Russell P, Quinn M, Ward B (1997) Tubal sterilisation, hysterectomy and decreased risk of ovarian cancer. Survey of Women's Health Study Group. *Int J Cancer* **71**: 948-951

Greenson JK, Isenhart CE, Rice R, Mojzisik C, Houchens D, Martin EW, Jr. (1994) Identification of occult micrometastases in pericolic lymph nodes of Duke's B colorectal cancer patients using monoclonal antibodies against cytokeratin and CC49. Correlation with long-term survival. *Cancer* **73**: 563-569

Gu J, Tamura M, Pankov R, Danen EH, Takino T, Matsumoto K, Yamada KM (1999) Shc and FAK differentially regulate cell motility and directionality modulated by

PTEN. *J Cell Biol* **146**: 389-403

Gubbay O, Guo W, Rae MT, Niven D, Langdon SP, Hillier SG (2005) Inflammation-associated gene expression is altered between normal human ovarian surface epithelial cells and cell lines derived from ovarian adenocarcinomas. *Br J Cancer* **92**: 1927-1933

Guidozzi F, Szumel RC, Ball JH, Johnston DA, Katz RL, Kidd L (1996) Image analysis of cellular DNA content in peritoneal fluid of patients with ovarian tumors of low malignant potential and invasive epithelial ovarian cancer. *Gynecol Oncol* **61**: 204-209

Hahn SA, Greenhalf B, Ellis I, Sina-Frey M, Rieder H, Korte B, Gerdes B, Kress R, Ziegler A, Raeburn JA, Campra D, Grutzmann R, Rehder H, Rothmund M, Schmiegel W, Neoptolemos JP, Bartsch DK (2003) BRCA2 germline mutations in familial pancreatic carcinoma. *J Natl Cancer Inst* **95**: 214-221

Hanahan D, Weinberg RA (2000) The hallmarks of cancer. *Cell* **100**: 57-70

Harris RE, Beebe-Donk J, Doss H, Burr DD (2005) Aspirin, ibuprofen, and other non-steroidal anti-inflammatory drugs in cancer prevention: a critical review of non-selective COX-2 blockade (review). *Oncol Rep* **13**: 559-583

Hartenbach EM, Olson TA, Goswitz JJ, Mohanraj D, Twiggs LB, Carson LF, Ramakrishnan S (1997) Vascular endothelial growth factor (VEGF) expression and survival in human epithelial ovarian carcinomas. *Cancer Lett* **121**: 169-175

Hartmann LC, Podratz KC, Keeney GL, Kamel NA, Edmonson JH, Grill JP, Su JQ, Katzmann JA, Roche PC (1994) Prognostic significance of p53 immunostaining in epithelial ovarian cancer. *J Clin Oncol* **12**: 64-69

Hatta Y, Takeuchi S, Yokota J, Koeffler HP (1997) Ovarian cancer has frequent loss of heterozygosity at chromosome 12p12.3-13.1 (region of TEL and Kip1 loci) and chromosome 12q23-ter: evidence for two new tumour-suppressor genes. *Br J Cancer* **75**: 1256-1262

Havrilesky LJ, Hurteau JA, Whitaker RS, Elbendary A, Wu S, Rodriguez GC, Bast RC, Jr., Berchuck A (1995) Regulation of apoptosis in normal and malignant ovarian epithelial cells by transforming growth factor beta. *Cancer Res* **55**: 944-948

Heaps JM, Nieberg RK, Berek JS (1990) Malignant neoplasms arising in endometriosis. *Obstet Gynecol* **75**: 1023-1028

Heid CA, Stevens J, Livak KJ, Williams PM (1996) Real time quantitative PCR. *Genome Res* **6**: 986-994

Heller DS, Gordon RE, Westhoff C, Gerber S (1996a) Asbestos exposure and ovarian fiber burden. *Am J Ind Med* **29**: 435-439

Heller DS, Westhoff C, Gordon RE, Katz N (1996b) The relationship between perineal cosmetic talc usage and ovarian talc particle burden. *Am J Obstet Gynecol* **174**: 1507-1510

Hellstrom I, Goodman G, Pullman J, Yang Y, Hellstrom KE (2001) Overexpression of HER-2 in ovarian carcinomas. *Cancer Res* **61**: 2420-2423

Helzlsouer KJ, Alberg AJ, Gordon GB, Longcope C, Bush TL, Hoffman SC, Comstock GW (1995) Serum gonadotropins and steroid hormones and the development of ovarian cancer. *JAMA* **274**: 1926-1930

Henriksen R, Funa K, Wilander E, Backstrom T, Ridderheim M, Oberg K (1993) Expression and prognostic significance of platelet-derived growth factor and its receptors in epithelial ovarian neoplasms. *Cancer Res* **53**: 4550-4554

Henriksen R, Wilander E, Oberg K (1995) Expression and prognostic significance of Bcl-2 in ovarian tumours. *Br J Cancer* **72**: 1324-1329

Henzen-Logmans SC, Berns EM, Klijn JG, van der Burg ME, Foekens JA (1992) Epidermal growth factor receptor in ovarian tumours: correlation of immunohistochemistry with ligand binding assay. *Br J Cancer* **66**: 1015-1021

Herod JJ, Eliopoulos AG, Warwick J, Niedobitek G, Young LS, Kerr DJ (1996) The prognostic significance of Bcl-2 and p53 expression in ovarian carcinoma. *Cancer Res* **56**: 2178-2184

Herrera CA, Xu L, Bucana CD, Silva e, V, Hess KR, Gershenson DM, Fidler IJ (2002) Expression of metastasis-related genes in human epithelial ovarian tumors. *Int J Oncol* **20**: 5-13

Higuchi R, Fockler C, Dollinger G, Watson R (1993) Kinetic PCR analysis: real-time monitoring of DNA amplification reactions. *Biotechnology (N Y)* **11**: 1026-1030

Hofmann J, Wegmann B, Hackenberg R, Kunzmann R, Schulz KD, Havemann K (1994) Production of insulin-like growth factor binding proteins by human ovarian carcinoma cells. *J Cancer Res Clin Oncol* **120**: 137-142

Hoggard N, Brintnell B, Howell A, Weissenbach J, Varley J (1995) Allelic imbalance on chromosome 1 in human breast cancer. II. Microsatellite repeat analysis. *Genes Chromosomes Cancer* **12**: 24-31

Hotte SJ, Winquist EW, Stitt L, Wilson SM, Chambers AF (2002) Plasma osteopontin: associations with survival and metastasis to bone in men with hormone-refractory prostate carcinoma. *Cancer* **95**: 506-512

Hough CD, Cho KR, Zonderman AB, Schwartz DR, Morin PJ (2001) Coordinately up-regulated genes in ovarian cancer. *Cancer Res* **61**: 3869-3876

Huddleston HG, Wong KK, Welch WR, Berkowitz RS, Mok SC (2005) Clinical applications of microarray technology: creatine kinase B is an up-regulated gene in epithelial ovarian cancer and shows promise as a serum marker. *Gynecol Oncol* **96**: 77-83

Hughes-Davies L, Huntsman D, Ruas M, Fuks F, Bye J, Chin SF, Milner J, Brown LA, Hsu F, Gilks B, Nielsen T, Schulzer M, Chia S, Ragaz J, Cahn A, Linger L, Ozdag H, Cattaneo E, Jordanova ES, Schuuring E, Yu DS, Venkitaraman A, Ponder B, Doherty A, Aparicio S, Bentley D, Theillet C, Ponting CP, Caldas C, Kouzarides T

(2003) EMSY links the BRCA2 pathway to sporadic breast and ovarian cancer. *Cell* **115**: 523-535

Hurteau J, Rodriguez GC, Whitaker RS, Shah S, Mills G, Bast RC, Berchuck A (1994) Transforming growth factor-beta inhibits proliferation of human ovarian cancer cells obtained from ascites. *Cancer* **74**: 93-99

Ichikawa Y, Yoshida S, Koyama Y, Hirai M, Ishikawa T, Nishida M, Tsunoda H, Kubo T, Miwa M, Uchida K (1996) Inactivation of p16/CDKN2 and p15/MTS2 genes in different histological types and clinical stages of primary ovarian tumors. *Int J Cancer* **69**: 466-470

Inoue K, Slaton JW, Eve BY, Kim SJ, Perrotte P, Balbay MD, Yano S, Bar-Eli M, Radinsky R, Pettaway CA, Dinney CP (2000) Interleukin 8 expression regulates tumorigenicity and metastases in androgen-independent prostate cancer. *Clin Cancer Res* **6**: 2104-2119

Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TP (2003) Summaries of Affymetrix GeneChip probe level data. *Nucleic Acids Res* **31**: e15

Ismail RS, Baldwin RL, Fang J, Browning D, Karlan BY, Gasson JC, Chang DD (2000) Differential gene expression between normal and tumor-derived ovarian epithelial cells. *Cancer Res* **60**: 6744-6749

Iwabuchi H, Sakamoto M, Sakunaga H, Ma YY, Carcangiu ML, Pinkel D, Yang-Feng TL, Gray JW (1995) Genetic analysis of benign, low-grade, and high-grade ovarian tumors. *Cancer Res* **55**: 6172-6180

Jacobs I, Bast RC, Jr. (1989) The CA 125 tumour-associated antigen: a review of the literature. *Hum Reprod* **4**: 1-12

Jacobs I, Davies AP, Bridges J, Stabile I, Fay T, Lower A, Grudzinskas JG, Oram D (1993) Prevalence screening for ovarian cancer in postmenopausal women by CA 125 measurement and ultrasonography. *BMJ* **306**: 1030-1034

Jacobs IJ, Kohler MF, Wiseman RW, Marks JR, Whitaker R, Kerns BA, Humphrey P, Berchuck A, Ponder BA, Bast RC, Jr. (1992) Clonal origin of epithelial ovarian carcinoma: analysis by loss of heterozygosity, p53 mutation, and X-chromosome inactivation. *J Natl Cancer Inst* **84**: 1793-1798

Jacobs IJ, Skates SJ, MacDonald N, Menon U, Rosenthal AN, Davies AP, Woolas R, Jeyarajah AR, Sibley K, Lowe DG, Oram DH (1999) Screening for ovarian cancer: a pilot randomised controlled trial. *Lancet* **353**: 1207-1210

Jazaeri AA, Lu K, Schmandt R, Harris CP, Rao PH, Sotiriou C, Chandramouli GV, Gershenson DM, Liu ET (2003) Molecular determinants of tumor differentiation in papillary serous ovarian carcinoma. *Mol Carcinog* **36**: 53-59

Jemal A, Murray T, Samuels A, Ghafoor A, Ward E, Thun MJ (2003) Cancer statistics, 2003. *CA Cancer J Clin* **53**: 5-26

Jimenez C, Jones DR, Rodriguez-Viciana P, Gonzalez-Garcia A, Leonardo E, Wennstrom S, von Kobbe C, Toran JL, Borlado L, Calvo V, Copin SG, Albar JP,

Gaspar ML, Diez E, Marcos MA, Downward J, Martinez A, Merida I, Carrera AC (1998) Identification and characterization of a new oncogene derived from the regulatory subunit of phosphoinositide 3-kinase. *EMBO J* **17**: 743-753

Joly DJ, Lilienfeld AM, Diamond EL, Bross ID (1974) An epidemiologic study of the relationship of reproductive experience to cancer of the ovary. *Am J Epidemiol* **99**: 190-209

Jones C, Foschini MP, Chaggar R, Lu YJ, Wells D, Shipley JM, Eusebi V, Lakhani SR (2000) Comparative genomic hybridization analysis of myoepithelial carcinoma of the breast. *Lab Invest* **80**: 831-836

Jones C, Nonni AV, Fulford L, Merrett S, Chaggar R, Eusebi V, Lakhani SR (2001) CGH analysis of ductal carcinoma of the breast with basaloid/myoepithelial cell differentiation. *Br J Cancer* **85**: 422-427

Jones PA, Buckley JD, Henderson BE, Ross RK, Pike MC (1991) From gene to carcinogen: a rapidly evolving field in molecular epidemiology. *Cancer Res* **51**: 3617-3620

Joukov V, Pajusola K, Kaipainen A, Chilov D, Lahtinen I, Kukk E, Saksela O, Kalkkinen N, Alitalo K (1996) A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. *EMBO J* **15**: 290-298

Jozan S, Guerrin M, Mazars P, Dutaur M, Monsarrat B, Cheutin F, Bugat R, Martel P, Valette A (1992) Transforming growth factor beta 1 (TGF-beta 1) inhibits growth of a human ovarian carcinoma cell line (OVCCR1) and is expressed in human ovarian tumors. *Int J Cancer* **52**: 766-770

Kaipainen A, Korhonen J, Mustonen T, van Hinsbergh VW, Fang GH, Dumont D, Breitman M, Alitalo K (1995) Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. *Proc Natl Acad Sci U S A* **92**: 3566-3570

Karasik A, Menczer J, Pariente C, Kanety H (1994) Insulin-like growth factor-I (IGF-I) and IGF-binding protein-2 are increased in cyst fluids of epithelial ovarian cancer. *J Clin Endocrinol Metab* **78**: 271-276

Katsaros D, Theillet C, Zola P, Louason G, Sanfilippo B, Isaia E, Arisio R, Giardina G, Sismondi P (1995) Concurrent abnormal expression of erbB-2, myc and ras genes is associated with poor outcome of ovarian cancer patients. *Anticancer Res* **15**: 1501-1510

KEAL EE (1960) Asbestosis and abdominal neoplasms. *Lancet* **2**: 1211-1216

Kennedy SG, Wagner AJ, Conzen SD, Jordan J, Bellacosa A, Tsichlis PN, Hay N (1997) The PI 3-kinase/Akt signaling pathway delivers an anti-apoptotic signal. *Genes Dev* **11**: 701-713

Kerr JF, Wyllie AH, Currie AR (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* **26**: 239-257

Khwaja A, Rodriguez-Viciana P, Wennstrom S, Warne PH, Downward J (1997) Matrix adhesion and Ras transformation both activate a phosphoinositide 3-OH kinase and protein kinase B/Akt cellular survival pathway. *EMBO J* **16**: 2783-2793

Kim JH, Skates SJ, Uede T, Wong Kk KK, Schorge JO, Feltmate CM, Berkowitz RS, Cramer DW, Mok SC (2002a) Osteopontin as a potential diagnostic biomarker for ovarian cancer. *JAMA* **287**: 1671-1679

Kim JH, Skates SJ, Uede T, Wong KK, Schorge JO, Feltmate CM, Berkowitz RS, Cramer DW, Mok SC (2002b) Osteopontin as a potential diagnostic biomarker for ovarian cancer. *JAMA* **287**: 1671-1679

Kim TM, Benedict WF, Xu HJ, Hu SX, Gosewehr J, Velicescu M, Yin E, Zheng J, D'Ablaing G, Dubeau L (1994) Loss of heterozygosity on chromosome 13 is common only in the biologically more aggressive subtypes of ovarian epithelial tumors and is associated with normal retinoblastoma gene expression. *Cancer Res* **54**: 605-609

Kinzler KW, Vogelstein B (1996) Lessons from hereditary colorectal cancer. *Cell* **87**: 159-170

Kitahara O, Furukawa Y, Tanaka T, Kihara C, Ono K, Yanagawa R, Nita ME, Takagi T, Nakamura Y, Tsunoda T (2001) Alterations of gene expression during colorectal carcinogenesis revealed by cDNA microarrays after laser-capture microdissection of tumor tissues and normal epithelia. *Cancer Res* **61**: 3544-3549

Kliewer EV, Smith KR (1995) Ovarian cancer mortality among immigrants in Australia and Canada. *Cancer Epidemiol Biomarkers Prev* **4**: 453-458

Klippel A, Escobedo MA, Wachowicz MS, Apell G, Brown TW, Giedlin MA, Kavanaugh WM, Williams LT (1998) Activation of phosphatidylinositol 3-kinase is sufficient for cell cycle entry and promotes cellular changes characteristic of oncogenic transformation. *Mol Cell Biol* **18**: 5699-5711

Knauf S, Urbach GI (1978) The development of a double-antibody radioimmunoassay for detecting ovarian tumor-associated antigen fraction OCA in plasma. *Am J Obstet Gynecol* **131**: 780-787

Knudson AG (1997) Hereditary predisposition to cancer. *Ann N Y Acad Sci* **833**: 58-67

Kohler M, Bauknecht T, Grimm M, Birmelin G, Kommoss F, Wagner E (1992) Epidermal growth factor receptor and transforming growth factor alpha expression in human ovarian carcinomas. *Eur J Cancer* **28A**: 1432-1437

Kommoss F, Wintzer HO, Von Kleist S, Kohler M, Walker R, Langton B, Van Tran K, Pfleiderer A, Bauknecht T (1990) In situ distribution of transforming growth factor alpha in normal human tissues and in malignant tumours of the ovary. *J Pathol* **162**: 223-230

Konecny G, Untch M, Pihan A, Kimmig R, Gropp M, Stieber P, Hepp H, Slamon D, Pegram M (2001) Association of urokinase-type plasminogen activator and its inhibitor with disease progression and prognosis in ovarian cancer. *Clin Cancer Res*

7: 1743-1749

Korkola JE, DeVries S, Fridlyand J, Hwang ES, Estep AL, Chen YY, Chew KL, Dairkee SH, Jensen RM, Waldman FM (2003) Differentiation of lobular versus ductal breast carcinomas by expression microarray analysis. *Cancer Res* **63**: 7167-7175

Kristiansen G, Denkert C, Schluns K, Dahl E, Pilarsky C, Hauptmann S (2002) CD24 is expressed in ovarian cancer and is a new independent prognostic marker of patient survival. *Am J Pathol* **161**: 1215-1221

Kudoh K, Ichikawa Y, Yoshida S, Hirai M, Kikuchi Y, Nagata I, Miwa M, Uchida K (2002) Inactivation of p16/CDKN2 and p15/MTS2 is associated with prognosis and response to chemotherapy in ovarian cancer. *Int J Cancer* **99**: 579-582

Kuhn W, Kaufmann M, Feichter GE, Rummel HH, Schmid H, Heberling D (1989) DNA flow cytometry, clinical and morphological parameters as prognostic factors for advanced malignant and borderline ovarian tumors. *Gynecol Oncol* **33**: 360-367

Kuhn W, Schmalfeldt B, Reuning U, Pache L, Berger U, Ulm K, Harbeck N, Spathe K, Dettmar P, Hofler H, Janicke F, Schmitt M, Graeff H (1999) Prognostic significance of urokinase (uPA) and its inhibitor PAI-1 for survival in advanced ovarian carcinoma stage FIGO IIIc. *Br J Cancer* **79**: 1746-1751

Kurose K, Zhou XP, Araki T, Cannistra SA, Maher ER, Eng C (2001) Frequent loss of PTEN expression is linked to elevated phosphorylated Akt levels, but not associated with p27 and cyclin D1 expression, in primary epithelial ovarian carcinomas. *Am J Pathol* **158**: 2097-2106

Lacey JV, Jr., Mink PJ, Lubin JH, Sherman ME, Troisi R, Hartge P, Schatzkin A, Schairer C (2002) Menopausal hormone replacement therapy and risk of ovarian cancer. *JAMA* **288**: 334-341

Lancaster JM, Dressman HK, Whitaker RS, Havrilesky L, Gray J, Marks JR, Nevins JR, Berchuck A (2004) Gene expression patterns that characterize advanced stage serous ovarian cancers. *J Soc Gynecol Investig* **11**: 51-59

Lane DP (1992) Cancer. p53, guardian of the genome. *Nature* **358**: 15-16

Launonen V, Stenback F, Puistola U, Bloigu R, Huusko P, Kytola S, Kauppila A, Winqvist R (1998) Chromosome 11q22.3-q25 LOH in ovarian cancer: association with a more aggressive disease course and involved subregions. *Gynecol Oncol* **71**: 299-304

Li C, Hung WW (2001) Model-based analysis of oligonucleotide arrays: model validation, design issues and standard error application. *Genome Biol* **2**: RESEARCH0032

Li S, Han H, Resnik E, Carcangiu ML, Schwartz PE, Yang-Feng TL (1993) Advanced ovarian carcinoma: molecular evidence of unifocal origin. *Gynecol Oncol* **51**: 21-25

Li SB, Schwartz PE, Lee WH, Yang-Feng TL (1991) Allele loss at the retinoblastoma locus in human ovarian cancer. *J Natl Cancer Inst* **83**: 637-640

Liang P, Pardee AB (1992) Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science* **257**: 967-971

Liotta LA (1992) Cancer cell invasion and metastasis. *Sci Am* **266**: 54-3

Liu JR, Fletcher B, Page C, Hu C, Nunez G, Baker V (1998) Bcl-xL is expressed in ovarian carcinoma and modulates chemotherapy-induced apoptosis. *Gynecol Oncol* **70**: 398-403

Loft A, Lidegaard O, Tabor A (1997) Incidence of ovarian cancer after hysterectomy: a nationwide controlled follow up. *Br J Obstet Gynaecol* **104**: 1296-1301

Lombardi D, Lacombe ML, Paggi MG (2000) nm23: unraveling its biological function in cell differentiation. *J Cell Physiol* **182**: 144-149

Lounis H, Mes-Masson AM, Dion F, Bradley WE, Seymour RJ, Provencher D, Tonin PN (1998) Mapping of chromosome 3p deletions in human epithelial ovarian tumors. *Oncogene* **17**: 2359-2365

Loupart ML, Armour J, Walker R, Adams S, Brammar W, Varley J (1995) Allelic imbalance on chromosome 1 in human breast cancer. I. Minisatellite and RFLP analysis. *Genes Chromosomes Cancer* **12**: 16-23

Lu KH, Weitzel JN, Kodali S, Welch WR, Berkowitz RS, Mok SC (1997) A novel 4-cM minimally deleted region on chromosome 11p15.1 associated with high grade nonmucinous epithelial ovarian carcinomas. *Cancer Res* **57**: 387-390

Luca M, Huang S, Gershenwald JE, Singh RK, Reich R, Bar-Eli M (1997) Expression of interleukin-8 by human melanoma cells up-regulates MMP-2 activity and increases tumor growth and metastasis. *Am J Pathol* **151**: 1105-1113

Lynch HT, Smyrk T (1996) Hereditary nonpolyposis colorectal cancer (Lynch syndrome). An updated review. *Cancer* **78**: 1149-1167

MacDonald TJ, Brown KM, LaFleur B, Peterson K, Lawlor C, Chen Y, Packer RJ, Cogen P, Stephan DA (2001) Expression profiling of medulloblastoma: PDGFRA and the RAS/MAPK pathway as therapeutic targets for metastatic disease. *Nat Genet* **29**: 143-152

Maehama T, Dixon JE (1998) The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem* **273**: 13375-13378

Maines-Bandiera SL, Auersperg N (1997) Increased E-cadherin expression in ovarian surface epithelium: an early step in metaplasia and dysplasia? *Int J Gynecol Pathol* **16**: 250-255

Makinen T, Jussila L, Veikkola T, Karpanen T, Kettunen MI, Pulkkanen KJ, Kauppinen R, Jackson DG, Kubo H, Nishikawa S, Yla-Herttuala S, Alitalo K (2001) Inhibition of lymphangiogenesis with resulting lymphedema in transgenic mice expressing soluble VEGF receptor-3. *Nat Med* **7**: 199-205

Mandai M, Konishi I, Koshiyama M, Mori T, Arao S, Tashiro H, Okamura H, Nomura

H, Hiai H, Fukumoto M (1994) Expression of metastasis-related nm23-H1 and nm23-H2 genes in ovarian carcinomas: correlation with clinicopathology, EGFR, c-erbB-2, and c-erbB-3 genes, and sex steroid receptor expression. *Cancer Res* **54**: 1825-1830

Mark HF, Afify AM, Werness BA, Das S, Mark S, Samy M (1999) Trisomy 8 in stage I and stage III ovarian cancer detected by fluorescence in situ hybridization. *Exp Mol Pathol* **66**: 76-81

Marks JR, Davidoff AM, Kerns BJ, Humphrey PA, Pence JC, Dodge RK, Clarke-Pearson DL, Iglehart JD, Bast RC, Jr., Berchuck A (1991) Overexpression and mutation of p53 in epithelial ovarian cancer. *Cancer Res* **51**: 2979-2984

Marth C, Lang T, Cronauer MV, Doppler W, Zeimet AG, Bachmair F, Ullrich A, Daxenbichler G (1992) Epidermal growth factor reduces HER-2 protein level in human ovarian carcinoma cells. *Int J Cancer* **52**: 311-316

Matei D, Graeber TG, Baldwin RL, Karlan BY, Rao J, Chang DD (2002) Gene expression in epithelial ovarian carcinoma. *Oncogene* **21**: 6289-6298

McGuckin MA, Cummings MC, Walsh MD, Hohn BG, Bennett IC, Wright RG (1996) Occult axillary node metastases in breast cancer: their detection and prognostic significance. *Br J Cancer* **73**: 88-95

McKenzie SJ, DeSombre KA, Bast BS, Hollis DR, Whitaker RS, Berchuck A, Boyer CM, Bast RC, Jr. (1993) Serum levels of HER-2 neu (C-erbB-2) correlate with overexpression of p185neu in human ovarian cancer. *Cancer* **71**: 3942-3946

Medl M, Ogris E, Peters-Engl C, Leodolter S (1995) TATI (tumour-associated trypsin inhibitor) as a marker of ovarian cancer. *Br J Cancer* **71**: 1051-1054

Meili R, Cron P, Hemmings BA, Ballmer-Hofer K (1998) Protein kinase B/Akt is activated by polyomavirus middle-T antigen via a phosphatidylinositol 3-kinase-dependent mechanism. *Oncogene* **16**: 903-907

Meinhold-Heerlein I, Bauerschlag D, Hilpert F, Dimitrov P, Sapinoso LM, Orlowska-Volk M, Bauknecht T, Park TW, Jonat W, Jacobsen A, Sehouli J, Luttges J, Krajewski M, Krajewski S, Reed JC, Arnold N, Hampton GM (2005) Molecular and prognostic distinction between serous ovarian carcinomas of varying grade and malignant potential. *Oncogene* **24**: 1053-1065

Mok CH, Tsao SW, Knapp RC, Fishbaugh PM, Lau CC (1992) Unifocal origin of advanced human epithelial ovarian cancers. *Cancer Res* **52**: 5119-5122

Mok SC, Chao J, Skates S, Wong K, Yiu GK, Muto MG, Berkowitz RS, Cramer DW (2001b) Prostasin, a potential serum marker for ovarian cancer: identification through microarray technology. *J Natl Cancer Inst* **93**: 1458-1464

Mok SC, Chao J, Skates S, Wong K, Yiu GK, Muto MG, Berkowitz RS, Cramer DW (2001a) Prostasin, a potential serum marker for ovarian cancer: identification through microarray technology. *J Natl Cancer Inst* **93**: 1458-1464

Mor G, Visintin I, Lai Y, Zhao H, Schwartz P, Rutherford T, Yue L, Bray-Ward P,

Ward DC (2005) Serum protein markers for early detection of ovarian cancer. *Proc Natl Acad Sci U S A* **102**: 7677-7682

Moreno-Bueno G, Sanchez-Estevez C, Cassia R, Rodriguez-Perales S, Diaz-Uriarte R, Dominguez O, Hardisson D, Andujar M, Prat J, Matias-Guiu X, Cigudosa JC, Palacios J (2003) Differential gene expression profile in endometrioid and nonendometrioid endometrial carcinoma: STK15 is frequently overexpressed and amplified in nonendometrioid carcinomas. *Cancer Res* **63**: 5697-5702

Morishige K, Kurachi H, Amemiya K, Fujita Y, Yamamoto T, Miyake A, Tanizawa O (1991) Evidence for the involvement of transforming growth factor alpha and epidermal growth factor receptor autocrine growth mechanism in primary human ovarian cancers in vitro. *Cancer Res* **51**: 5322-5328

Moser TL, Young TN, Rodriguez GC, Pizzo SV, Bast RC, Jr., Stack MS (1994) Secretion of extracellular matrix-degrading proteinases is increased in epithelial ovarian carcinoma. *Int J Cancer* **56**: 552-559

Muller A, Homey B, Soto H, Ge N, Catron D, Buchanan ME, McClanahan T, Murphy E, Yuan W, Wagner SN, Barrera JL, Mohar A, Verastegui E, Zlotnik A (2001) Involvement of chemokine receptors in breast cancer metastasis. *Nature* **410**: 50-56

Muller JM, Metzger E, Greschik H, Bosserhoff AK, Mercep L, Buettner R, Schule R (2002) The transcriptional coactivator FHL2 transmits Rho signals from the cell membrane into the nucleus. *EMBO J* **21**: 736-748

Murdoch WJ, Townsend RS, McDonnel AC (2001) Ovulation-induced DNA damage in ovarian surface epithelial cells of ewes: prospective regulatory mechanisms of repair/survival and apoptosis. *Biol Reprod* **65**: 1417-1424

Myers MP, Stolarov JP, Eng C, Li J, Wang SI, Wigler MH, Parsons R, Tonks NK (1997) P-TEN, the tumor suppressor from human chromosome 10q23, is a dual-specificity phosphatase. *Proc Natl Acad Sci U S A* **94**: 9052-9057

Naidu R, Wahab NA, Yadav M, Kutty MK (2002) Protein expression and molecular analysis of c-myc gene in primary breast carcinomas using immunohistochemistry and differential polymerase chain reaction. *Int J Mol Med* **9**: 189-196

Narod S, Ford D, Devilee P, Barkardottir RB, Eyfjord J, Lenoir G, Serova O, Easton D, Goldgar D (1995a) Genetic heterogeneity of breast-ovarian cancer revisited. Breast Cancer Linkage Consortium. *Am J Hum Genet* **57**: 957-958

Narod SA, Ford D, Devilee P, Barkardottir RB, Lynch HT, Smith SA, Ponder BA, Weber BL, Garber JE, Birch JM, . (1995b) An evaluation of genetic heterogeneity in 145 breast-ovarian cancer families. Breast Cancer Linkage Consortium. *Am J Hum Genet* **56**: 254-264

Negus RP, Stamp GW, Relf MG, Burke F, Malik ST, Bernasconi S, Allavena P, Sozzani S, Mantovani A, Balkwill FR (1995) The detection and localization of monocyte chemoattractant protein-1 (MCP-1) in human ovarian cancer. *J Clin Invest* **95**: 2391-2396

Nelson AR, Fingleton B, Rothenberg ML, Matrisian LM (2000) Matrix

metalloproteinases: biologic activity and clinical implications. *J Clin Oncol* **18**: 1135-1149

Newhouse ML, Pearson RM, Fullerton JM, Boesen EA, Shannon HS (1977) A case control study of carcinoma of the ovary. *Br J Prev Soc Med* **31**: 148-153

Niederacher D, Yan HY, An HX, Bender HG, Beckmann MW (1999) CDKN2A gene inactivation in epithelial sporadic ovarian cancer. *Br J Cancer* **80**: 1920-1926

Obata K, Hoshiai H (2000) Common genetic changes between endometriosis and ovarian cancer. *Gynecol Obstet Invest* **50 Suppl 1**: 39-43

Okubo K, Hori N, Matoba R, Niiyama T, Fukushima A, Kojima Y, Matsubara K (1992) Large scale cDNA sequencing for analysis of quantitative and qualitative aspects of gene expression. *Nat Genet* **2**: 173-179

Ono K, Tanaka T, Tsunoda T, Kitahara O, Kihara C, Okamoto A, Ochiai K, Takagi T, Nakamura Y (2000) Identification by cDNA microarray of genes involved in ovarian carcinogenesis. *Cancer Res* **60**: 5007-5011

Ooka M, Sakita I, Fujiwara Y, Tamaki Y, Yamamoto H, Aihara T, Miyazaki M, Kadota M, Masuda N, Sugita Y, Iwao K, Monden M (2000) Selection of mRNA markers for detection of lymph node micrometastases in breast cancer patients. *Oncol Rep* **7**: 561-566

Owens OJ, Stewart C, Brown I, Leake RE (1991a) Epidermal growth factor receptors (EGFR) in human ovarian cancer. *Br J Cancer* **64**: 907-910

Owens OJ, Stewart C, Leake RE (1991b) Growth factors in ovarian cancer. *Br J Cancer* **64**: 1177-1181

Owens OJ, Stewart C, Leake RE, McNicol AM (1992) A comparison of biochemical and immunohistochemical assessment of EGFR expression in ovarian cancer. *Anticancer Res* **12**: 1455-1458

Ozalp S, Yalcin OT, Gulbas Z, Tanir HM, Minsin T (2001) Effect of cellular DNA content on the prognosis of epithelial ovarian cancers. *Gynecol Obstet Invest* **52**: 93-97

Paget S (1889) The distribution of secondary growths in cancer of the breast. *Lancet* **1**: 99-101

Paley PJ, Staskus KA, Gebhard K, Mohanraj D, Twiggs LB, Carson LF, Ramakrishnan S (1997) Vascular endothelial growth factor expression in early stage ovarian carcinoma. *Cancer* **80**: 98-106

Parazzini F, Franceschi S, La Vecchia C, Fasoli M (1991) The epidemiology of ovarian cancer. *Gynecol Oncol* **43**: 9-23

Patton SE, Martin ML, Nelsen LL, Fang X, Mills GB, Bast RC, Jr., Ostrowski MC (1998) Activation of the ras-mitogen-activated protein kinase pathway and phosphorylation of ets-2 at position threonine 72 in human ovarian cancer cell lines.

Cancer Res **58**: 2253-2259

Pennica D, Swanson TA, Welsh JW, Roy MA, Lawrence DA, Lee J, Brush J, Taneyhill LA, Deuel B, Lew M, Watanabe C, Cohen RL, Melhem MF, Finley GG, Quirke P, Goddard AD, Hillan KJ, Gurney AL, Botstein D, Levine AJ (1998) WISP genes are members of the connective tissue growth factor family that are up-regulated in wnt-1-transformed cells and aberrantly expressed in human colon tumors. *Proc Natl Acad Sci U S A* **95**: 14717-14722

Perou CM, Jeffrey SS, van de RM, Rees CA, Eisen MB, Ross DT, Pergamenschikov A, Williams CF, Zhu SX, Lee JC, Lashkari D, Shalon D, Brown PO, Botstein D (1999) Distinctive gene expression patterns in human mammary epithelial cells and breast cancers. *Proc Natl Acad Sci U S A* **96**: 9212-9217

Purdie DM, Bain CJ, Siskind V, Russell P, Hacker NF, Ward BG, Quinn MA, Green AC (1999) Hormone replacement therapy and risk of epithelial ovarian cancer. *Br J Cancer* **81**: 559-563

Ramaswamy S, Ross KN, Lander ES, Golub TR (2003) A molecular signature of metastasis in primary solid tumors. *Nat Genet* **33**: 49-54

Ramaswamy S, Tamayo P, Rifkin R, Mukherjee S, Yeang CH, Angelo M, Ladd C, Reich M, Latulippe E, Mesirov JP, Poggio T, Gerald W, Loda M, Lander ES, Golub TR (2001) Multiclass cancer diagnosis using tumor gene expression signatures. *Proc Natl Acad Sci U S A* **98**: 15149-15154

Rangel LB, Agarwal R, D'Souza T, Pizer ES, Alo PL, Lancaster WD, Gregoire L, Schwartz DR, Cho KR, Morin PJ (2003) Tight junction proteins claudin-3 and claudin-4 are frequently overexpressed in ovarian cancer but not in ovarian cystadenomas. *Clin Cancer Res* **9**: 2567-2575

Relaix F, Wei XJ, Wu X, Sassoon DA (1998) Peg3/Pw1 is an imprinted gene involved in the TNF-NFkappaB signal transduction pathway. *Nat Genet* **18**: 287-291

Reles A, Wen WH, Schmider A, Gee C, Runnebaum IB, Kilian U, Jones LA, el Naggar A, Minguillon C, Schonborn I, Reich O, Kreienberg R, Lichtenegger W, Press MF (2001) Correlation of p53 mutations with resistance to platinum-based chemotherapy and shortened survival in ovarian cancer. *Clin Cancer Res* **7**: 2984-2997

Resnicoff M, Ambrose D, Coppola D, Rubin R (1993) Insulin-like growth factor-1 and its receptor mediate the autocrine proliferation of human ovarian carcinoma cell lines. *Lab Invest* **69**: 756-760

Riman T, Dickman PW, Nilsson S, Correia N, Nordlinder H, Magnusson CM, Weiderpass E, Persson IR (2002) Hormone replacement therapy and the risk of invasive epithelial ovarian cancer in Swedish women. *J Natl Cancer Inst* **94**: 497-504

Risch HA, Howe GR (1995) Pelvic inflammatory disease and the risk of epithelial ovarian cancer. *Cancer Epidemiol Biomarkers Prev* **4**: 447-451

Risch HA, McLaughlin JR, Cole DE, Rosen B, Bradley L, Kwan E, Jack E, Vesprini DJ, Kuperstein G, Abrahamson JL, Fan I, Wong B, Narod SA (2001) Prevalence and

penetrance of germline BRCA1 and BRCA2 mutations in a population series of 649 women with ovarian cancer. *Am J Hum Genet* **68**: 700-710

Risinger JI, Berchuck A, Kohler MF, Boyd J (1994) Mutations of the E-cadherin gene in human gynecologic cancers. *Nat Genet* **7**: 98-102

Rosenblatt KA, Thomas DB (1996) Reduced risk of ovarian cancer in women with a tubal ligation or hysterectomy. The World Health Organization Collaborative Study of Neoplasia and Steroid Contraceptives. *Cancer Epidemiol Biomarkers Prev* **5**: 933-935

Ross DT, Scherf U, Eisen MB, Perou CM, Rees C, Spellman P, Iyer V, Jeffrey SS, van de RM, Waltham M, Pergamenschikov A, Lee JC, Lashkari D, Shalon D, Myers TG, Weinstein JN, Botstein D, Brown PO (2000) Systematic variation in gene expression patterns in human cancer cell lines. *Nat Genet* **24**: 227-235

Rossing MA, Daling JR, Weiss NS, Moore DE, Self SG (1994) Ovarian tumors in a cohort of infertile women. *N Engl J Med* **331**: 771-776

Rubin SC, Finstad CL, Wong GY, Almadrones L, Plante M, Lloyd KO (1993) Prognostic significance of HER-2/neu expression in advanced epithelial ovarian cancer: a multivariate analysis. *Am J Obstet Gynecol* **168**: 162-169

Ryan A, Al Jehani RM, Mulligan KT, Jacobs IJ (1998) No evidence exists for methylation inactivation of the p16 tumor suppressor gene in ovarian carcinogenesis. *Gynecol Oncol* **68**: 14-17

Salazar H, Godwin AK, Daly MB, Laub PB, Hogan WM, Rosenblum N, Boente MP, Lynch HT, Hamilton TC (1996) Microscopic benign and invasive malignant neoplasms and a cancer-prone phenotype in prophylactic oophorectomies. *J Natl Cancer Inst* **88**: 1810-1820

Sambrook J, Gething MJ (1989) Protein structure. Chaperones, paperones. *Nature* **342**: 224-225

Santin AD, Zhan F, Bellone S, Palmieri M, Cane S, Bignotti E, Anfossi S, Gokden M, Dunn D, Roman JJ, O'Brien TJ, Tian E, Cannon MJ, Shaughnessy J, Jr., Pecorelli S (2004) Gene expression profiles in primary ovarian serous papillary tumors and normal ovarian epithelium: identification of candidate molecular markers for ovarian cancer diagnosis and therapy. *Int J Cancer* **112**: 14-25

Saretzki G, Hoffmann U, Rohlke P, Psille R, Gaigal T, Keller G, Hofler H, Loning T, Petersen I, Dietel M (1997) Identification of allelic losses in benign, borderline, and invasive epithelial ovarian tumors and correlation with clinical outcome. *Cancer* **80**: 1241-1249

Sato K, Qian J, Slezak JM, Lieber MM, Bostwick DG, Bergstralh EJ, Jenkins RB (1999) Clinical significance of alterations of chromosome 8 in high-grade, advanced, nonmetastatic prostate carcinoma. *J Natl Cancer Inst* **91**: 1574-1580

Sawiris GP, Sherman-Baust CA, Becker KG, Cheadle C, Teichberg D, Morin PJ (2002) Development of a highly specialized cDNA array for the study and diagnosis

of epithelial ovarian cancer. *Cancer Res* **62**: 2923-2928

Scherf U, Ross DT, Waltham M, Smith LH, Lee JK, Tanabe L, Kohn KW, Reinhold WC, Myers TG, Andrews DT, Scudiero DA, Eisen MB, Sausville EA, Pommier Y, Botstein D, Brown PO, Weinstein JN (2000) A gene expression database for the molecular pharmacology of cancer. *Nat Genet* **24**: 236-244

Schildkraut JM, Bastos E, Berchuck A (1997) Relationship between lifetime ovulatory cycles and overexpression of mutant p53 in epithelial ovarian cancer. *J Natl Cancer Inst* **89**: 932-938

Schueler JA, Trimbos JB, vd BM, Cornelisse CJ, Hermans J, Fleuren GJ (1996) DNA index reflects the biological behavior of ovarian carcinoma stage I-IIa. *Gynecol Oncol* **62**: 59-66

Schummer M, Ng WV, Bumgarner RE, Nelson PS, Schummer B, Bednarski DW, Hassell L, Baldwin RL, Karlan BY, Hood L (1999) Comparative hybridization of an array of 21,500 ovarian cDNAs for the discovery of genes overexpressed in ovarian carcinomas. *Gene* **238**: 375-385

Schwab M, Amler LC (1990) Amplification of cellular oncogenes: a predictor of clinical outcome in human cancer. *Genes Chromosomes Cancer* **1**: 181-193

Scotton CJ, Wilson JL, Milliken D, Stamp G, Balkwill FR (2001) Epithelial cancer cell migration: a role for chemokine receptors? *Cancer Res* **61**: 4961-4965

Scully RE (1995) Early de novo ovarian cancer and cancer developing in benign ovarian lesions. *Int J Gynaecol Obstet* **49 Suppl**: S9-15

Sellar GC, Watt KP, Rabiasz GJ, Stronach EA, Li L, Miller EP, Massie CE, Miller J, Contreras-Moreira B, Scott D, Brown I, Williams AR, Bates PA, Smyth JF, Gabra H (2003) OPCML at 11q25 is epigenetically inactivated and has tumor-suppressor function in epithelial ovarian cancer. *Nat Genet* **34**: 337-343

Session DR, Lee GS, Choi J, Wolgemuth DJ (1999) Expression of cyclin E in gynecologic malignancies. *Gynecol Oncol* **72**: 32-37

Sgroi DC, Teng S, Robinson G, LeVangie R, Hudson JR, Jr., Elkahloun AG (1999) In vivo gene expression profile analysis of human breast cancer progression. *Cancer Res* **59**: 5656-5661

Shay JW (1998) Telomerase in cancer: diagnostic, prognostic, and therapeutic implications. *Cancer J Sci Am* **4 Suppl 1**: S26-S34

Shayesteh L, Lu Y, Kuo WL, Baldocchi R, Godfrey T, Collins C, Pinkel D, Powell B, Mills GB, Gray JW (1999) PIK3CA is implicated as an oncogene in ovarian cancer. *Nat Genet* **21**: 99-102

Shen N, Weiderpass E, Antilla A, Goldberg MS, Vasama-Neuvonen KM, Boffetta P, Vainio HU, Partanen TJ (1998) Epidemiology of occupational and environmental risk factors related to ovarian cancer. *Scand J Work Environ Health* **24**: 175-182

Shetye J, Christensson B, Rubio C, Rodensjo M, Biberfeld P, Mellstedt H (1989) The

tumor-associated antigens BR55-2, GA73-3 and GICA 19-9 in normal and corresponding neoplastic human tissues, especially gastrointestinal tissues. *Anticancer Res* **9**: 395-404

Shigemasa K, Tanimoto H, Underwood LJ, Parmley TH, Arihiro K, Ohama K, O'Brien TJ (2001) Expression of the protease inhibitor antileukoprotease and the serine protease stratum corneum chymotryptic enzyme (SCCE) is coordinated in ovarian tumors. *Int J Gynecol Cancer* **11**: 454-461

Shridhar V, Lee J, Pandita A, Iturria S, Avula R, Staub J, Morrissey M, Calhoun E, Sen A, Kalli K, Keeney G, Roche P, Cliby W, Lu K, Schmandt R, Mills GB, Bast RC, Jr., James CD, Couch FJ, Hartmann LC, Lillie J, Smith DI (2001) Genetic analysis of early- versus late-stage ovarian tumors. *Cancer Res* **61**: 5895-5904

Shu XO, Brinton LA, Gao YT, Yuan JM (1989) Population-based case-control study of ovarian cancer in Shanghai. *Cancer Res* **49**: 3670-3674

Shvartsman HS, Lu KH, Lee J, Lillie J, Deavers MT, Clifford S, Wolf JK, Mills GB, Bast RC, Jr., Gershenson DM, Schmandt R (2003) Overexpression of kallikrein 10 in epithelial ovarian carcinomas. *Gynecol Oncol* **90**: 44-50

Simone G, Falco G, Caponio MA, Campobasso C, De Frenza M, Petroni S, Wiesel S, Leone A (2001) nm23 expression in malignant ascitic effusions of serous ovarian adenocarcinoma. *Int J Oncol* **19**: 885-890

Simone NL, Bonner RF, Gillespie JW, Emmert-Buck MR, Liotta LA (1998) Laser-capture microdissection: opening the microscopic frontier to molecular analysis. *Trends Genet* **14**: 272-276

Singh D, Febbo PG, Ross K, Jackson DG, Manola J, Ladd C, Tamayo P, Renshaw AA, D'Amico AV, Richie JP, Lander ES, Loda M, Kantoff PW, Golub TR, Sellers WR (2002) Gene expression correlates of clinical prostate cancer behavior. *Cancer Cell* **1**: 203-209

Skates SJ, Xu FJ, Yu YH, Sjovall K, Einhorn N, Chang Y, Bast RC, Jr., Knapp RC (1995) Toward an optimal algorithm for ovarian cancer screening with longitudinal tumor markers. *Cancer* **76**: 2004-2010

Skirnisdottir I, Sorbe B, Karlsson M, Seidal T (2001) Prognostic importance of DNA ploidy and p53 in early stages of epithelial ovarian carcinoma. *Int J Oncol* **19**: 1295-1302

Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de RM, Jeffrey SS, Thorsen T, Quist H, Matese JC, Brown PO, Botstein D, Eystein LP, Borresen-Dale AL (2001) Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci U S A* **98**: 10869-10874

Stacker SA, Caesar C, Baldwin ME, Thornton GE, Williams RA, Prevo R, Jackson DG, Nishikawa S, Kubo H, Achen MG (2001) VEGF-D promotes the metastatic spread of tumor cells via the lymphatics. *Nat Med* **7**: 186-191

Steichen-Gersdorf E, Gallion HH, Ford D, Girodet C, Easton DF, DiCioccio RA,

Evans G, Ponder MA, Pye C, Mazoyer S, . (1994) Familial site-specific ovarian cancer is linked to BRCA1 on 17q12-21. *Am J Hum Genet* **55**: 870-875

Steinbach D, Hermann J, Viehmann S, Zintl F, Gruhn B (2002) Clinical implications of PRAME gene expression in childhood acute myeloid leukemia. *Cancer Genet Cytogenet* **133**: 118-123

Stephan C, Yousef GM, Scorilas A, Jung K, Jung M, Kristiansen G, Hauptmann S, Kishi T, Nakamura T, Loening SA, Diamandis EP (2004) Hepsin is highly over expressed in and a new candidate for a prognostic indicator in prostate cancer. *J Urol* **171**: 187-191

Stratton JF, Thompson D, Bobrow L, Dalal N, Gore M, Bishop DT, Scott I, Evans G, Daly P, Easton DF, Ponder BA (1999) The genetic epidemiology of early-onset epithelial ovarian cancer: a population-based study. *Am J Hum Genet* **65**: 1725-1732

Stromberg K, Johnson GR, O'Connor DM, Sorensen CM, Gullick WJ, Kannan B (1994) Frequent immunohistochemical detection of EGF supergene family members in ovarian carcinogenesis. *Int J Gynecol Pathol* **13**: 342-347

Struewing JP, Hartge P, Wacholder S, Baker SM, Berlin M, McAdams M, Timmerman MM, Brody LC, Tucker MA (1997) The risk of cancer associated with specific mutations of BRCA1 and BRCA2 among Ashkenazi Jews. *N Engl J Med* **336**: 1401-1408

Su AI, Cooke MP, Ching KA, Hakak Y, Walker JR, Wiltshire T, Orth AP, Vega RG, Sapinoso LM, Moqrich A, Patapoutian A, Hampton GM, Schultz PG, Hogenesch JB (2002) Large-scale analysis of the human and mouse transcriptomes. *Proc Natl Acad Sci U S A* **99**: 4465-4470

Su AI, Welsh JB, Sapinoso LM, Kern SG, Dimitrov P, Lapp H, Schultz PG, Powell SM, Moskaluk CA, Frierson HF, Jr., Hampton GM (2001) Molecular classification of human carcinomas by use of gene expression signatures. *Cancer Res* **61**: 7388-7393

Sundfeldt K, Piontkewitz Y, Ivarsson K, Nilsson O, Hellberg P, Brannstrom M, Janson PO, Enerback S, Hedin L (1997) E-cadherin expression in human epithelial ovarian cancer and normal ovary. *Int J Cancer* **74**: 275-280

Suzuki M, Ohwada M, Sato I, Nagatomo M (1995) Serum level of macrophage colony-stimulating factor as a marker for gynecologic malignancies. *Oncology* **52**: 128-133

Szala S, Froehlich M, Scollon M, Kasai Y, Steplewski Z, Koprowski H, Linnenbach AJ (1990) Molecular cloning of cDNA for the carcinoma-associated antigen GA733-2. *Proc Natl Acad Sci U S A* **87**: 3542-3546

Tai YT, Lee S, Niloff E, Weisman C, Strobel T, Cannistra SA (1998) BAX protein expression and clinical outcome in epithelial ovarian cancer. *J Clin Oncol* **16**: 2583-2590

Tamayo P, Slonim D, Mesirov J, Zhu Q, Kitareewan S, Dmitrovsky E, Lander ES, Golub TR (1999) Interpreting patterns of gene expression with self-organizing maps:

methods and application to hematopoietic differentiation. *Proc Natl Acad Sci U S A* **96**: 2907-2912

Tamura M, Gu J, Takino T, Yamada KM (1999a) Tumor suppressor PTEN inhibition of cell invasion, migration, and growth: differential involvement of focal adhesion kinase and p130Cas. *Cancer Res* **59**: 442-449

Tamura M, Gu J, Tran H, Yamada KM (1999b) PTEN gene and integrin signaling in cancer. *J Natl Cancer Inst* **91**: 1820-1828

Tanimoto H, Yan Y, Clarke J, Korourian S, Shigemasa K, Parmley TH, Parham GP, O'Brien TJ (1997) Hepsin, a cell surface serine protease identified in hepatoma cells, is overexpressed in ovarian cancer. *Cancer Res* **57**: 2884-2887

Tapper J, Kettunen E, El Rifai W, Seppala M, Andersson LC, Knuutila S (2001) Changes in gene expression during progression of ovarian carcinoma. *Cancer Genet Cytogenet* **128**: 1-6

Tas F, Tuzlali S, Aydiner A, Saip P, Salihoglu Y, Iplikci A, Topuz E (2002) Prognostic role of nm23 gene expression in patients with ovarian cancer. *Am J Clin Oncol* **25**: 164-167

Tavassoli M, Steingrimsdottir H, Pierce E, Jiang X, Alagoz M, Farzaneh F, Campbell IG (1996) Loss of heterozygosity on chromosome 5q in ovarian cancer is frequently accompanied by TP53 mutation and identifies a tumour suppressor gene locus at 5q13.1-21. *Br J Cancer* **74**: 115-119

Tavazoie S, Hughes JD, Campbell MJ, Cho RJ, Church GM (1999) Systematic determination of genetic network architecture. *Nat Genet* **22**: 281-285

Tonin PN, Hudson TJ, Rodier F, Bossolasco M, Lee PD, Novak J, Manderson EN, Provencher D, Mes-Masson AM (2001) Microarray analysis of gene expression mirrors the biology of an ovarian cancer model. *Oncogene* **20**: 6617-6626

Tsuda H, Takarabe T, Hasegawa F, Fukutomi T, Hirohashi S (2000) Large, central acellular zones indicating myoepithelial tumor differentiation in high-grade invasive ductal carcinomas as markers of predisposition to lung and brain metastases. *Am J Surg Pathol* **24**: 197-202

Tung KH, Wilkens LR, Wu AH, McDuffie K, Nomura AM, Kolonel LN, Terada KY, Goodman MT (2005) Effect of anovulation factors on pre- and postmenopausal ovarian cancer risk: revisiting the incessant ovulation hypothesis. *Am J Epidemiol* **161**: 321-329

Ueda M, Terai Y, Kumagai K, Ueki K, Yamaguchi H, Akise D, Ueki M (2001) Vascular endothelial growth factor C gene expression is closely related to invasion phenotype in gynecological tumor cells. *Gynecol Oncol* **82**: 162-166

van Dam PA, Vergote IB, Lowe DG, Watson JV, van Damme P, van der Auwera JC, Shepherd JH (1994) Expression of c-erbB-2, c-myc, and c-ras oncoproteins, insulin-like growth factor receptor I, and epidermal growth factor receptor in ovarian carcinoma. *J Clin Pathol* **47**: 914-919

van der Burg ME, Henzen-Logmans SC, Berns EM, van Putten WL, Klijn JG, Foekens JA (1996) Expression of urokinase-type plasminogen activator (uPA) and its inhibitor PAI-1 in benign, borderline, malignant primary and metastatic ovarian tumors. *Int J Cancer* **69**: 475-479

van NJ, Jr., DePriest PD, Puls LE, Donaldson ES, Gallion HH, Pavlik EJ, Powell DE, Kryscio RJ (1991) Ovarian cancer screening in asymptomatic postmenopausal women by transvaginal sonography. *Cancer* **68**: 458-462

van NJ, Jr., DePriest PD, Reedy MB, Gallion HH, Ueland FR, Pavlik EJ, Kryscio RJ (2000) The efficacy of transvaginal sonographic screening in asymptomatic women at risk for ovarian cancer. *Gynecol Oncol* **77**: 350-356

van NJ, Jr., Higgins RV, Donaldson ES, Gallion HH, Powell DE, Pavlik EJ, Woods CH, Thompson EA (1990) Transvaginal sonography as a screening method for ovarian cancer. A report of the first 1000 cases screened. *Cancer* **65**: 573-577

Varambally S, Dhanasekaran SM, Zhou M, Barrette TR, Kumar-Sinha C, Sanda MG, Ghosh D, Pienta KJ, Sewalt RG, Otte AP, Rubin MA, Chinnaiyan AM (2002) The polycomb group protein EZH2 is involved in progression of prostate cancer. *Nature* **419**: 624-629

Velculescu VE, Zhang L, Vogelstein B, Kinzler KW (1995) Serial analysis of gene expression. *Science* **270**: 484-487

Venn A, Watson L, Bruinsma F, Giles G, Healy D (1999) Risk of cancer after use of fertility drugs with in-vitro fertilisation. *Lancet* **354**: 1586-1590

Venter PF (1981) Ovarian epithelial cancer and chemical carcinogenesis. *Gynecol Oncol* **12**: 281-285

Verschraegen CF, Hu W, Du Y, Mendoza J, Early J, Deavers M, Freedman RS, Bast RC, Jr., Kudelka AP, Kavanagh JJ, Giovanella BC (2003) Establishment and characterization of cancer cell cultures and xenografts derived from primary or metastatic Mullerian cancers. *Clin Cancer Res* **9**: 845-852

Versnel MA, Haarbrink M, Langerak AW, de Laat PA, Hagemeijer A, van der Kwast TH, van den Berg-Bakker LA, Schrier PI (1994) Human ovarian tumors of epithelial origin express PDGF in vitro and in vivo. *Cancer Genet Cytogenet* **73**: 60-64

Vessey MP, Painter R (1995) Endometrial and ovarian cancer and oral contraceptives--findings in a large cohort study. *Br J Cancer* **71**: 1340-1342

Wan M, Li WZ, Duggan BD, Felix JC, Zhao Y, Dubeau L (1997) Telomerase activity in benign and malignant epithelial ovarian tumors. *J Natl Cancer Inst* **89**: 437-441

Wang J, Mes-Masson AM, Tonin PN, Provencher D, Eydoux P (2000) Trisomy of chromosome 10 in two cases of ovarian carcinoma. *Cancer Genet Cytogenet* **118**: 65-68

Wang K, Gan L, Jeffery E, Gayle M, Gown AM, Skelly M, Nelson PS, Ng WV, Schummer M, Hood L, Mulligan J (1999) Monitoring gene expression profile changes

in ovarian carcinomas using cDNA microarray. *Gene* **229**: 101-108

Watson MA, Darrow C, Zimonjic DB, Popescu NC, Fleming TP (1998) Structure and transcriptional regulation of the human mammaglobin gene, a breast cancer associated member of the uteroglobin gene family localized to chromosome 11q13. *Oncogene* **16**: 817-824

Watson MA, Fleming TP (1996) Mammaglobin, a mammary-specific member of the uteroglobin gene family, is overexpressed in human breast cancer. *Cancer Res* **56**: 860-865

Webb PM, Green A, Cummings MC, Purdie DM, Walsh MD, Chenevix-Trench G (1998) Relationship between number of ovulatory cycles and accumulation of mutant p53 in epithelial ovarian cancer. *J Natl Cancer Inst* **90**: 1729-1734

Wehner AP (2002) Cosmetic talc should not be listed as a carcinogen: comments on NTP's deliberations to list talc as a carcinogen. *Regul Toxicol Pharmacol* **36**: 40-50

Weigang B, Nap M, Bittl A, Jaeger W (1994) Immunohistochemical localization of insulin-like growth factor 1 receptors in benign and malignant tissues of the female genital tract. *Tumour Biol* **15**: 236-246

Welsh JB, Zarrinkar PP, Sapinoso LM, Kern SG, Behling CA, Monk BJ, Lockhart DJ, Burger RA, Hampton GM (2001) Analysis of gene expression profiles in normal and neoplastic ovarian tissue samples identifies candidate molecular markers of epithelial ovarian cancer. *Proc Natl Acad Sci U S A* **98**: 1176-1181

Wen X, Fuhrman S, Michaels GS, Carr DB, Smith S, Barker JL, Somogyi R (1998) Large-scale temporal gene expression mapping of central nervous system development. *Proc Natl Acad Sci U S A* **95**: 334-339

Whittemore AS, Gong G, Itnyre J (1997) Prevalence and contribution of BRCA1 mutations in breast cancer and ovarian cancer: results from three U.S. population-based case-control studies of ovarian cancer. *Am J Hum Genet* **60**: 496-504

Whittemore AS, Harris R, Itnyre J (1992) Characteristics relating to ovarian cancer risk: collaborative analysis of 12 US case-control studies. II. Invasive epithelial ovarian cancers in white women. Collaborative Ovarian Cancer Group. *Am J Epidemiol* **136**: 1184-1203

Whittemore AS, Wu ML, Paffenbarger RS, Jr., Sarles DL, Kampert JB, Grosser S, Jung DL, Ballon S, Hendrickson M, Mohle-Boetani J (1989) Epithelial ovarian cancer and the ability to conceive. *Cancer Res* **49**: 4047-4052

Wong C, Hempling RE, Piver MS, Natarajan N, Mettlin CJ (1999) Perineal talc exposure and subsequent epithelial ovarian cancer: a case-control study. *Obstet Gynecol* **93**: 372-376

Wright K, Wilson P, Morland S, Campbell I, Walsh M, Hurst T, Ward B, Cummings M, Chenevix-Trench G (1999) beta-catenin mutation and expression analysis in ovarian cancer: exon 3 mutations and nuclear translocation in 16% of endometrioid tumours. *Int J Cancer* **82**: 625-629

Wu R, Lin L, Beer DG, Ellenson LH, Lamb BJ, Rouillard JM, Kuick R, Hanash S, Schwartz DR, Fearon ER, Cho KR (2003) Amplification and overexpression of the L-MYC proto-oncogene in ovarian carcinomas. *Am J Pathol* **162**: 1603-1610

Wyllie AH, Kerr JF, Currie AR (1980) Cell death: the significance of apoptosis. *Int Rev Cytol* **68**: 251-306

Xu F, Yu Y, Le XF, Boyer C, Mills GB, Bast RC, Jr. (1999) The outcome of heregulin-induced activation of ovarian cancer cells depends on the relative levels of HER-2 and HER-3 expression. *Clin Cancer Res* **5**: 3653-3660

Xu FJ, Stack S, Boyer C, O'Briant K, Whitaker R, Mills GB, Yu YH, Bast RC, Jr. (1997) Heregulin and agonistic anti-p185(c-erbB2) antibodies inhibit proliferation but increase invasiveness of breast cancer cells that overexpress p185(c-erbB2): increased invasiveness may contribute to poor prognosis. *Clin Cancer Res* **3**: 1629-1634

Xu Y, Shen Z, Wiper DW, Wu M, Morton RE, Elson P, Kennedy AW, Belinson J, Markman M, Casey G (1998) Lysophosphatidic acid as a potential biomarker for ovarian and other gynecologic cancers. *JAMA* **280**: 719-723

Yang-Feng TL, Li S, Han H, Schwartz PE (1992) Frequent loss of heterozygosity on chromosomes Xp and 13q in human ovarian cancer. *Int J Cancer* **52**: 575-580

Yiu GK, Chan WY, Ng SW, Chan PS, Cheung KK, Berkowitz RS, Mok SC (2001) SPARC (secreted protein acidic and rich in cysteine) induces apoptosis in ovarian cancer cells. *Am J Pathol* **159**: 609-622

Yokoyama Y, Charnock-Jones DS, Licence D, Yanaihara A, Hastings JM, Holland CM, Emoto M, Umemoto M, Sakamoto T, Sato S, Mizunuma H, Smith SK (2003) Vascular endothelial growth factor-D is an independent prognostic factor in epithelial ovarian carcinoma. *Br J Cancer* **88**: 237-244

Young TN, Rodriguez GC, Rinehart AR, Bast RC, Jr., Pizzo SV, Stack MS (1996) Characterization of gelatinases linked to extracellular matrix invasion in ovarian adenocarcinoma: purification of matrix metalloproteinase 2. *Gynecol Oncol* **62**: 89-99

Yu D, Wolf JK, Scanlon M, Price JE, Hung MC (1993) Enhanced c-erbB-2/neu expression in human ovarian cancer cells correlates with more severe malignancy that can be suppressed by E1A. *Cancer Res* **53**: 891-898

Yu Y, Xu F, Peng H, Fang X, Zhao S, Li Y, Cuevas B, Kuo WL, Gray JW, Siciliano M, Mills GB, Bast RC, Jr. (1999) NOEY2 (ARHI), an imprinted putative tumor suppressor gene in ovarian and breast carcinomas. *Proc Natl Acad Sci U S A* **96**: 214-219

Zembutsu H, Ohnishi Y, Tsunoda T, Furukawa Y, Katagiri T, Ueyama Y, Tamaoki N, Nomura T, Kitahara O, Yanagawa R, Hirata K, Nakamura Y (2002) Genome-wide cDNA microarray screening to correlate gene expression profiles with sensitivity of 85 human cancer xenografts to anticancer drugs. *Cancer Res* **62**: 518-527

Zenklusen JC, Weitzel JN, Ball HG, Conti CJ (1995) Allelic loss at 7q31.1 in human primary ovarian carcinomas suggests the existence of a tumor suppressor gene.

Oncogene **11**: 359-363

Zhou Y, Gwadry FG, Reinhold WC, Miller LD, Smith LH, Scherf U, Liu ET, Kohn KW, Pommier Y, Weinstein JN (2002) Transcriptional regulation of mitotic genes by camptothecin-induced DNA damage: microarray analysis of dose- and time-dependent effects. *Cancer Res* **62**: 1688-1695

Zurawski VR, Jr., Orjaseter H, Andersen A, Jellum E (1988) Elevated serum CA 125 levels prior to diagnosis of ovarian neoplasia: relevance for early detection of ovarian cancer. *Int J Cancer* **42**: 677-680